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GRADUATE EDUCATION
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APPROVAL SHEET

Title of Dissertation: "In Vivo T Cell Signaling Leading to Apoptosis vs. Cytokine Production"

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Doctor of Philosophy Degree
17 August 1995

Dissertation and Abstract Approved:

Committee Chairperson	March 18, 1996 Date
Committee Member	Aug. 17 1995 Date
Committee Member	3/18/96 Date
Committee Member	3/18/96 Date
Committee Member	3/18/96 Date

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1996		**00-00-1996 to 00-00-1996**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
In Vivo T Cell Signalling Leading to Apoptosis vs. Cytokine Secretion	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Uniformed Services University of the Health Sciences,F. Edward Hebert School of Medicine,4301 Jones Bridge Road,Bethesda,MD,20814-4799**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
see report

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**		**218**	

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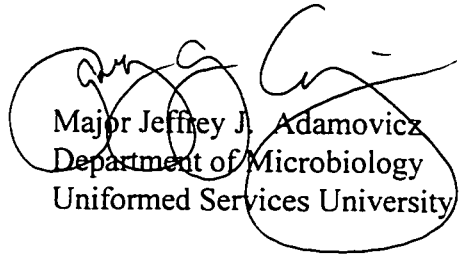

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ABSTRACT

Title of Dissertation: *In Vivo* T cell Signaling Leading to Apoptosis vs. Cytokine Production.

Major Jeffrey J. Adamovicz, Doctor of Philosophy, 1996.

Dissertation Directed By: Dr. William Gause, Associate Professor, Department of Microbiology.

Two signals are hypothesized as necessary for the activation of T cells leading to cytokine production. The first signal is delivered by the T cell receptor (TCR) and the second by a costimulatory signal, the best defined being CD28 and its homologue CTLA-4 (cytotoxic T lymphocyte associated protein-four). Treatment of T cells with antibodies against the invariant CD3-ϵ chain of the TCR-CD3 complex is a widely used model for T cell activation and is being considered as an immune suppressant for clinical trials, since mitogenic anti-CD3 antibodies administered *in vivo* induce T cell tolerance and depletion. Although extensive studies have been performed with anti-CD3 antibodies, little is known concerning the mechanisms involved in either anti-CD3 antibody-induced cytokine production or subsequent T cell depletion *in vivo*. Previous studies have suggested three possible fates for T cells stimulated by anti-CD3 antibodies: TCR blockade or modulation, functional nonresponsiveness or anergy, and apoptosis. In this investigation three assays were adapted to detect apoptosis of peripheral T cells. My findings suggest that anti-CD3 antibody-induced peripheral T cell depletion results from apoptosis following high but not low dose administration of anti-CD3 antibodies. This high density TCR ligation is sufficient to rapidly promote T cell differentiation to cytokine secretion in the absence of CD28/CTLA-

4 costimulatory signals. At lower anti-CD3 antibody doses T cell cytokine production is dependent on CTLA-4 ligands, suggesting that quantitative differences in TCR signaling influence the requirement for these costimulatory signals. The rapid expression of cytokines was detected in both $CD4^+$, $NK1.1^-$ and $CD4^-$, $NK1.1^+$ T cells. In addition anti-CD3 antibody-induced deletion of $CD8^+$ but not $CD4^+$ T cells was determined to be IL-2-dependent.

The anti-CD3 antibody-induced apoptosis was also found to be associated with the increased expression of *fas* and *fasL* and altered expression of cell surface markers on apoptotic cells, including the appearance of an unusual phenotype characterized by increased expression of B220 and decreased expression of CD4 and CD8: a phenotype also characteristic of the *fas* defective *lpr* mouse. These results suggest a possible common phenotype for T cells committed to undergo apoptosis.

IN VIVO T CELL SIGNALING LEADING TO APOPTOSIS VS. CYTOKINE SECRETION

by

Major Jeffrey J. Adamovicz

Dissertation submitted to the Faculty of the

Department of Microbiology Graduate Program of the

Uniformed Services University of the Health

Sciences in partial fulfillment of the degree of

Doctor of Philosophy 1996

ACKNOWLEDGMENTS

I would first like to thank the United States Army and the United States taxpayers for providing me with this fantastic opportunity to " be all I can be". This opportunity is without parallel and I will endeavor to prove that your confidence was not misplaced. I would also like to thank the numerous people both inside and outside the university whose reagents, ideas and advise have made this project possible. In particular, I would like to thank Dr. Fred Finkelman for his generous donation of antibodies, Mr. Mark Moorman for his expert technical assistance with FACS analysis and my Committee Chair Dr. Lee Metcalf as well as the other committee members, (Dr. William Gause, Dr. Susan Langreth, Dr. Dave Ledney and Dr. Jimmy Mond), for expediting the project. Last, and most importantly I would like to thank my family, especially my wife Brenda, my daughter Laura Alyse and my son Mason Ryder. They have paid for this Ph.D. in currency which can never be repaid: time and patience.

A Half Dozen Quotes

1) "Of the book of life, a little I can read" -Some famous guy

2) "The highest possible stage in moral culture is when we recognize that we ought to control our thoughts." -Charles Darwin

3) "If our soldiers are not overburdened with money, it is not because they have a distaste for riches; if their lives are not unduly long, it is not because they are disinclined to longevity." -Sun Tzu

4) "I believe, if we take habitual drunkards as a class, their heads and their hearts will bear an advantageous comparison with those of any other class. There seems ever to have been a proneness in the brilliant and warm-blooded to fall into this vice."-Abraham Lincoln

5) "It's just a job. Grass grows, birds fly, waves pound the sand. I beat people up." -Muhammad Ali

ABBREVIATIONS:

ADCC-antibody-dependent cell-mediated cytotoxicity

AICD-activation induced cell death

AMCA-7-amino-4-methyl coumarin-3-acetic acid

AO-acridine orange

APC-antigen presenting cell/allophycocyanin

ATA-aurin tricarboxylic acid

bp-base pair

CD-cluster of differentiation

CHO-Chinese hamster-ovary

CTL-cytotoxic T lymphocyte

CTLA-4-cytotoxic T lymphocyte-associated protein

D-day

DTH-delayed type hypersensitivity

EAE-experimental autoimmune encephalomyelitis

EB-ethidium bromide

EDTA-Ethylenediaminetetraacetic acid

FACS-fluorescence activated cell sorter

FcR-Fc-receptor

FITC-fluorescein isothiocyanate

FLANUL-fluorescent labeling and nick translation dUTP labeling

FSC-forward scatter

g-gram

g-gravity

GALT-gut-associated lymphoid tissue

h-hour

HEPES-*N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

HBSS-Hank's balanced salt solution

HPRT-hypoxanthine-guanine phosphoribosyl transferase

HSA-heat stable antigen

ICAM-1 intercellular cell adhesion molecule

IFN-interferon

Ig-immunoglobin

IL-interleukin

iv- intravenous

L-liter

LCMV-Lymphocytic Choriomeningitis virus

LFA-1-lymphocyte function-related antigen-1

LMP-low melting point

LPS-lipopolysaccharide

M-molar

MBP-myelin basic protein

mg-milligram

MHC-major histocompatibility complex

$MgCL_2$-magnesium chloride

ml-milliliter

mm-millimeter

nMol-nanomoles

μMol-micromoles

NaCL-sodium chloride

NaOH-sodium hydroxide

NCI-National Cancer Institute

ng-nanogram

NIH-National Institutes of Health

NK-natural killer

nm-nanometer

OD-optical density

PBMC-peripheral blood mononuclear cell

PBS-phosphate-buffered saline

PCR-polymerase chain reaction

PE-phycoerythrin

PI-phosphatidylinositol

PKC-protein kinase C

PMA-phorbal myristate acid

PTK-protein tyrosine kinase

RBC-red blood cell

RNA-ribonucleic acid

RT-reverse transcription

SA-streptavidin

SEA-Staphylococcal enterotoxin A

SEB-Staphylococcal enterotoxin B

SEM-standard error of the mean

SDS-sodium dodecyl sulphate

SLE-Serum Lupus Erythematosus

SSC-side scatter

S.S.DNA-salmon-sperm deoxyribonucleic acid

TBE-Tris Base EDTA

TCR-T cell receptor

TE-Tris EDTA

Th1/Th2-T helper 1, T helper 2

TNF-tumor necrosis factor

TUNEL-TdT-mediated dUTP-biotin nick end labeling

µg-microgram

µl-microliter

µm-micrometer

UV-ultraviolet

vs.-versus

V-volt

VSV-vesicular stomatitis virus

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Introduction.

Regulation of T cell activation: a primer. T cell homeostasis is a dynamic process requiring a balance between the rapid activation of antigen-specific cells and the inactivation of any autoreactive T cells. A two-signal model of T cell activation was originally proposed by Bretscher and Cohn more than twenty-five years ago (Bretscher and Cohn.1970). This model proposed that two distinct signals were required to fully activate antigen-specific T/B cells. The first signal was delivered via the cell's antigen-specific receptor and the second signal by some other cell to cell interaction. Subsequently this model has been substantiated: the first signal was demonstrated to be a cognate signal which is antigen-specific and MHC-restricted and is provided through the T cell receptor (TCR), and the second or costimulatory-signal is provided through a nonantigen-specific signal. Perhaps the best described T cell costimulatory receptor is CD28, although a number of other cell-surface and secreted costimulatory molecules have been proposed. These putative T cell signaling molecules include, but are not limited to, those with proposed costimulatory signaling capability: CD2, CD40L, heat stable antigen (HSA), CD27 and CTLA4 (Clark and Ledbetter.1994; Liu *et al.*, 1992; Liu *et al.*, 1992; Johnson and Jenkins.1994; June *et al.*, 1994; Hintzen *et al.*, 1995); those that augment cell-cell interactions, which include integrins and adhesion molecules ICAM-1, LFA-1 and VLA-4 (Clark and Ledbetter.1994; Damle *et al.*, 1993); cytokines including IL-1, IL-4 or the combination of IL-2, TNF-α and IL-6 (McArthur and Raulet.1993; Unutmaz *et al.*, 1994; Lichtman *et al.*, 1988). The most potent T cell costimulatory signaling molecule, CD28, and also its homolog, CTLA-4, bind the cell-surface molecules B7-1 (CD80) and B7-2 (CD86), which are expressed on antigen

presenting cells (APCs) including dendritic cells and activated but not resting B cells and macrophages (Koulova *et al.*,1991; Linsley *et al.*,1991; Linsley and Ledbetter.1993; June *et al.*,1994). Signaling through the TCR and the costimulatory molecule CD28 is sufficient for T cell activation *in vitro* and required for T cell activation *in vivo* (Linsley *et al.*,1991; Clark and Ledbetter.1994; Jenkins *et al.*,1990; Linsley and Ledbetter.1993).

A second outcome of TCR-signaling was suggested through *in vitro* and *in vivo* studies which demonstrated that the provision of signal one through anti-CD3 antibodies or superantigens in the absence of signal two can lead to a state of antigen-specific nonresponsiveness or anergy in T cells (Jenkins.1992; Schwartz.1993; Weiss.1993; Hirsch *et al.*,1988; Hughes *et al.*,1994; Henrickson *et al.*,1994; Herold *et al.*,1992). Anergic T cells fail to produce autocrine growth factors (IL-2/IL-4) and are unresponsive to subsequent antigenic challenge (DeSilva *et al.*,1991; Schwartz.1990). The anergy seen *in vitro* when T cells are stimulated with anti-CD3 or anti-TCR antibodies can be prevented by the addition of stimulatory antibodies to CD28 (Harding *et al.*,1992; June *et al.*,1994). Following the induction of anergy *in vitro*, cells assume a state of growth arrest; these cells can sometimes be rescued through the subsequent provision of exogenous IL-2 in combination with the restimulation of the TCR, which is illustrative of the role that certain cytokines play in T cell activation (June *et al.*,1990; Jenkins.1992).

A third possible fate following signaling through the TCR was suggested from *in vivo* studies demonstrating depletion of antigen-specific T cells following high-dose antigen administration and other studies showing depletion of peripheral T cells inappropriately activated with bacterial superantigens. The depletion of peripheral T cells in both cases was

hypothesized as activation-induced cell death (AICD) (Critchfield *et al.*, 1994; Moskophidis *et al.*, 1993; Carlow *et al.*, 1992; Mamalaki *et al.*, 1993; Kawabe and Ochi. 1991; MacDonald *et al.*, 1991). Although studies with superantigens are useful for studying activation-induced T cell depletion, they have been complicated by several factors. These studies demonstrated that each superantigen is limited in that it affects only a small subset of Vβ specific T cells and within this subset there is a differential response, *i.e.*, some cells are activated and are deleted; other cells become anergic and in some target cells no effect is observed (Scott *et al.*, 1993; Gonzalo *et al.*, 1992; Webb *et al.*, 1994). Most importantly, these studies failed to define the mechanism of deletion or to develop adequate techniques for detecting AICD *in vivo*.

A good model for studying T cell activation is antibody-induced signaling through the CD3ϵ chain of the TCR complex. Anti-CD3 antibodies can bind to and signal most cells expressing a CD3 complex and is thus able to mimic cognate antigen stimulation in the majority of T cells. The activation of peripheral T cells in the absence of proper costimulation is believed to be a mechanism whereby autoimmune responses may be avoided through the induction of AICD (Schwartz. 1993). Since autoimmunity is relatively rare, this suggests that there may be costimulatory dependent regulatory mechanisms to prevent nonspecific activation of T cells. The use of anti-CD3 antibodies in an *in vivo* model of T cell activation may provide a better understanding of the mechanism of AICD, provide an increased understanding of autoimmune disease, and may also lead to increased insights into control of graft rejection.

Use of anti-CD3 antibody as an initiator of TCR signaling in T cells. Models using antibodies directed at the TCR complex to mimic cognate-antigen binding have been widely used to study T cell activation and tolerance induction both *in vitro* and *in vivo* (Weiss.1993). The TCR is a multi-chain complex on the surface of T cells which confers antigen-specificity. The chains which make up the receptor include: the polymorphic α and β chains which form the heterologous dimer that recognizes peptide antigens bound to the major histocompatibility complex (MHC), the invariant CD3 complex consisting of γ, δ and two ϵ chains, and one pair of either ζ or η chains or a heterologous pair of one of each chain (Weiss.1993). Cross-linking the T cell receptor through antibodies directed at the CD3 complex has been shown to induce rapid tyrosine phosphorylation of the γ, δ, ϵ and ζ chains through the cytoplasmic protein-tyrosine kinases (PTKs). These phosphorylation events lead to the subsequent phosphatidylinositol (PI) hydrolysis, protein kinase C (PKC) activation, and subsequent IL-2 production (Weiss.1993; Mueller *et al.*, 1989). These events were found to be biologically relevant as pre-treatment with herbimycin A, which inhibits PTK, and cholera toxin, which blocks PI hydrolysis, were shown to block IL-2 production in both anti-CD3 antibody-stimulated and cognate antigen-stimulated T cells (Migata *et al.*, 1994). The anti-CD3 antibody, 145-2C11, used for these studies, is effective as a TCR signal transducer as it can bind to and stimulate cells expressing a TCR/CD3 complex (Leo *et al.*, 1987).

Dose-dependent activation of naive T cells *via* plate bound anti-CD3 antibody causes a prolonged state of nonresponsiveness or anergy unless these celis are also provided costimulation by APCs expressing B7 (Seder *et al.*, 1994; Linsley *et al.*, 1991; Harding *et*

al.,1992; Chiodetti and Schwartz.1992; Chen and Nabavi.1994; Reiser *et al.*,1992). The stimulation of activated memory T cells, hybridomas, or T cell lines with plate bound anti-CD3 antibody leads to AICD of these cells which also appears to be dose-dependent. The same treatment of resting memory T cells with anti-CD3 antibody leads to activation and proliferation, suggesting that the activation and maturation state of a T cell may determine the outcome of anti-CD3 antibody stimulation (Ucker *et al.*,1992; Russell *et al.*,1991; Sarin *et al.*,1993; Luqman and Bottomly.1992; Wesselborg *et al.*,1993; Lightstone *et al.*,1993). These findings are supported by recent *in vivo* experiments suggesting that naive B cells can not activate naive T cells due to a lack of costimulatory ligands; but can activate resting memory T cells independently of costimulatory signals (Ronchese and Hausmann.1993; Morris *et al.*,1994).

The administration of antibodies against the CD3 complex *in vivo* leads to a different outcome. In mice and humans, treatment with high doses of anti-CD3 antibodies induces immune suppression associated with an initial peripheral T cell activation, followed by depletion of peripheral T cells (Hirsch *et al.*,1989). It was also demonstrated that treatment of mice with high doses of anti-CD3 antibody led to peripheral T cell depletion and suppression of skin graft rejection by altering TCR expression in the remaining T cells (Hirsch *et al.*,1988). These results suggest anti-CD3 antibody-induced T cell depletion and tolerance induction may occur by multiple mechanisms including: 1) stearic hindrance of the TCR/MHC interaction by antibodies directed at the TCR/CD3 complex or modulation of the TCR complex by cross-linking and capping; 2) T cell dysfunction through the induction of

anergy; 3) T cell depletion through AICD or apoptosis; 4) T cell migration; 5) Complement-mediated cytotoxicity or antibody-dependent cell-mediated cytotoxicity (ADCC).

These mechanisms of T cell depletion following anti-CD3 antibody treatment *in vivo* are not mutually exclusive and there is some evidence to support all of them. Evidence that differentiates among the first three possibilities has been obtained from *in vivo* studies. *In vivo* treatment with anti-CD3 antibodies reduces the peripheral T cells in the blood and lymphoid organs in a dose-dependent manner. This reduction has been attributed in part to the observed TCR modulation and extravasation of the T cells suggesting that temporary modulation of the TCR may be the cause of anti-CD3 induced T cell depletion (Hirsch *et al.*, 1988; Davis *et al.*, 1989). This mechanism seems unlikely because it would require maintenance of T cells that do not express TCRs in the periphery for weeks to months. Anti-CD3 antibody and SEB treatments both lead to an activation-induced depletion of peripheral T cells *in vivo* following signaling via the TCR; which requires the interaction of an FcR^- APC for the former and an MHC class II^- cell in the later treatment. SEB induced T cell depletion has been demonstrated to be through AICD (Russell *et al.*, 1991; Shi *et al.*, 1991). *In vivo* studies with thymectomized superantigen-treated mice have demonstrated that recovery of superantigen-responsiveness in surviving SEB responsive T cells is due to the reversal of anergy and not the re-expression of the TCR (Migita and Ochi. 1993). In addition, it was recently shown that the response to superantigens can be predicted by the initial activation event, *i.e.*, staphylococcal enterotoxin B (SEB)-responsive cells which proliferate undergo apoptosis while those that do not proliferate become anergic, suggesting that

proliferation is probably a required step toward AICD (Renno *et al.*, 1995). These later events may also occur in anti-CD3 antibody-treated mice.

Anergy has been suggested as a mechanism of immune suppression following treatment with nonmitogenic anti-CD3 antibodies. This treatment was not associated with T cell depletion or modulation of the T cell receptor, suggesting that nonmitogenic anti-CD3 antibody may induce anergy rather than apoptosis (Hughes *et al.*, 1994; Herold *et al.*, 1992). The *in vivo* induction of cytokines, but not suppression of graft rejection, has been shown to require the interaction of a FcγR-bearing cell. Mitogenic-IgG anti-CD3 antibodies induced morbidity and mortality, production of the cytokines TNF-α, IFN-γ and GM-CSF, as well as an anti-CD3 antibody humoral immune response and a suppressed graft rejection. The induction of cytokines and anti-CD3 humoral antibody responses were greatly reduced in mice treated with $F(ab')_2$ antibodies, while suppression of allograft rejection was maintained, suggesting the importance of the FcγR-bearing cell in determining the outcome of anti-CD3 antibody-induced T cell activation (Hirsch *et al.*, 1990; Woodle *et al.*, 1991; Hirsch *et al.*, 1991; Hirsch *et al.*, 1991). These findings were strengthened by experiments in mice and humans by switching the isotype of the anti-CD3 antibody from a FcγR binding isotype to a non-FcγR binding isotype which resulted in inhibition of cytokine production and prevention of graft *vs.* host disease in the human studies and autoimmune diabetes in mice (Herold *et al.*, 1992; Anasetti *et al.*, 1992; Parren *et al.*, 1992; Alegre *et al.*, 1994; Woodle *et al.*, 1991). Together these data suggest that the binding of anti-CD3 antibody to a FcγR on an APC is important in determining the outcome of TCR signaling. It appears that the APC interaction facilitates a deletion mechanism when mediating anti-CD3 cross-linking. The

APC may be required simply as a platform for TCR cross-linking or for the provision of other signals; which leads to apoptosis. In the absence of APC-mediated cross-linking or other signaling events, anti-CD3 antibody binding appears to be sufficient to induce anergy.

It was hypothesized that a large number of T cells may undergo anti-CD3 antibody-induced deletion following *in vivo* treatment, although there is, as yet, little evidence to support this possibility in the periphery (Hirsch *et al.*, 1988; Groux *et al.*, 1993; Hirsch *et al.*, 1991; Janssen *et al.*, 1991). Evidence does support an apoptotic mechanism in the thymus following anti-CD3 antibody treatment both *in vitro* (Smith *et al.*, 1989) and *in vivo* (Shi *et al.*, 1991). In a separate *in vitro* study, evidence was presented to support a model of anti-CD3 antibody-induced negative selection following high doses of antibody, and positive selection after exposure to low dose anti-CD3 antibody treatment (Iwata *et al.*, 1991). These data suggest that anti-CD3 antibody signaling can mimic cognate TCR signaling and supports the notion of positive selection of thymocytes after a weak TCR signal and negative selection through apoptosis following a strong TCR signal. Additional evidence that anti-CD3 antibody can mimic cognate TCR signaling was demonstrated by the ability of protein-tyrosine kinase inhibitors to block anti-CD3, but not glucocorticoid-induced apoptosis (Migata *et al.*, 1994). These data suggest that thymocyte apoptosis following anti-CD3 antibody-mediated signaling requires TCR-derived signaling and subsequent phosphorylation events at a sufficient level to initiate apoptosis. *In vivo* studies with mitogenic anti-CD3 antibodies should serve as a model to study the mechanism of activation-induced peripheral T cell depletion.

Upon activation, T cells may acquire the ability to migrate from the circulation and lymphoid tissues. T cells which are activated with alloantigens can migrate to the gut-associated lymphoid tissue (GALT) where a significant number of the migrating cells are subsequently extruded into the gut lumen (Sprent.1976). Therefore, T cell migration cannot be ruled out as an explanation for the T cell depletion caused by high dose anti-CD3 antibody administration. The classical complement-mediated mechanism is also unlikely to explain the depletion of peripheral T cells because experiments in which the isotype of a murine anti-human anti-CD3 antibody was switched from IgG2a to IgG1 or IgG2b revealed a differential ability to induce T cell depletion (Parren *et al.*, 1992; Anasetti *et al.*, 1992; Alegre *et al.*, 1994; Woodle*et al.*, 1991; Ceuppens *et al.*, 1985; Woodle *et al.*, 1991). All three murine anti-human anti-CD3 antibody isotypes can activate the classical complement pathway but only IgG2a leads to T cell depletion, which suggests that complement-mediated cell lysis is not the reason for T cell depletion in this model. In mice, the $F(ab')_2$ fragments of hamster IgG antibody was shown to induce anergy rather than depletion *in vivo,* suggesting that depletion requires the interaction of a FcγR-bearing cell for subsequent cell-to-cell signaling or possibly to mediate ADCC (Herold *et al.*, 1992; Hirsch *et al.*, 1990; Hirsch *et al.*, 1991; Hirsch *et al.*, 1991). The commonly used anti-CD3 antibody, 145-2C11, is a hamster IgG antibody which can redirect $CD8^+$ CTLs to mediate lysis of FcR-bearing cells, but it is not known whether this antibody could stimulate the murine-effector mechanisms of complement-mediated lysis or ADCC against $CD3^+$ T cells (Leo *et al.*, 1987). Therefore, these mechanisms cannot be ruled out as the cause of anti-CD3 antibody-induced depletion.

T cell activation leading to deletion; the role of costimulation. Activation of a mature T cell requires proper signaling through the TCR and costimulatory ligand CD28. If properly activated, a peripheral T cell may differentiate from a naive resting cell to a cytokine producing effector. Alternatively, activation of a mature T cell in the absence of costimulation can lead to elimination of the responding T cell.

T cells can be nonspecifically activated *in vitro* with mitogens like the phorbol ester. phorbol myristate acetate (PMA) or lectins such as concanavalin A combined with the calcium ionophore ionomycin, but the activation of T cells in a physiological manner requires two signals. These two signals must include signaling through the TCR and a costimulatory signal through CD28 or CTLA4 (June *et al.*, 1994). Evidence to suggest that CD28 is an obligate costimulatory molecule for T cell activation was first shown when monoclonal antibodies to CD28 in conjunction with plate-bound anti-TCR or anti-CD3 antibodies could costimulate the production of multiple cytokines (June *et al.*, 1990; Harding *et al.*, 1992; Norton *et al.*, 1992). By blocking the CD28 ligands CD80 and CD86 separately, with anti-B7-1 or B7-2 antibodies respectively, or simultaneously with the fusion protein CTLA4-Ig, it was clearly demonstrated that T cell activation was dependent on CD28-mediated signaling (Koulova *et al.*, 1991; Linsley *et al.*, 1991; Jenkins. 1994). The *in vivo* significance of CD28 costimulation was demonstrated with CD-28 knockout mice. These mice had no IL-2 response to lectins, greatly reduced circulating immunoglobulin levels and impaired Ig-class switching after infection with vesicular stomatitis virus (VSV), all of which suggested an impairment in T helper function. These mice were able to mount a DTH response and produce CTLs after infection with lymphocytic choriomeningitis virus

(LCMV), suggesting that the CD28 costimulatory pathway is not required for all T cell responses *in vivo* (Shahinian *et al.*, 1993). It was subsequently demonstrated *in vitro* that T lymphocytes from CD28 knockout mice had greatly suppressed anti-CD3 antibody and alloantigen-induced proliferation, even in the presence of induced CTLA4 expression, suggesting that CD28 is the primary T cell costimulatory molecule (Green *et al.*, 1994). The induction T cell anergy *in vitro* in the absence of CD28 costimulation may illustrate a primary control mechanism for the prevention of nonspecific T cell activation *in vivo* and ultimately the prevention of autoimmunity.

A second T cell costimulatory molecule that is homologous to CD28 was subsequently identified on the surface of activated T cells and was designated CTLA4. This ligand, as opposed to CD28, is not expressed constitutively but only after TCR signaling, although its avidity is 20 times that of CD28 for B7 (Linsley *et al.*, 1991; June *et al.*, 1994). The existence of CTLA4 may explain the costimulatory-dependent responses observed in CD28 knockout mice (Green *et al.*, 1994). Although CTLA4 can provide T cell costimulation, an additional negative T cell regulatory role has been proposed. *In vitro* experiments with anti-CTLA4 antibodies, in the presence of anti-CD3 and anti-CD28 antibodies, demonstrated an inhibition of T cell proliferation (Walunas *et al.*, 1994). An experiment with cultured antigen-activated human T cells demonstrated that CTLA4 cross-linking led to AICD, which implicated CTLA4 as a negative costimulatory signal (Gribben *et al.*, 1995).

The ligand for CD28/CTLA4, B7, was first defined by its ability to costimulate T cell proliferation and increase IL-2 gene expression (Norton *et al.*, 1992; Fraser and Weiss. 1992;

Schwartz.1992). To prove that T cell costimulation was mediated by B7-dependent ligation, anti-CD28 or anti-B7 antibodies were used to block B7-transfected Chinese hamster ovary (CHO) cell mediated T cell costimulation (June *et al.*,1994; Linsley and Ledbetter.1993). The results of studies with B7 knockout mice indicated a lack of immune defects, suggesting the presence of more than one costimulatory ligand for CD28/CTLA4. Experiments demonstrating that the fusion protein CTLA4-Ig was able to suppress graft rejection, while anti-B7 antibodies could not, strongly suggested an alternative CD28 ligand. Subsequently a second B7 family member, B7-2 (CD86), was isolated (June *et al.*,1994; Linsley and Ledbetter.1993). Taken together, these studies suggest that proper T cell activation requires costimulation through the CD28/CTLA4 costimulatory molecules on T cells by binding with B7-1 and/or B7-2 on APCs and that this costimulation can be blocked by the fusion protein CTLA4-Ig. The addition of a chimeric CTLA4-Ig protein prevents T cells from responding to cognate antigen/MHC stimulation both *in vitro* and *in vivo* through its prevention of CTLA-4/CD28 co-receptor interactions with B7 ligands on APC (Linsley *et al.*,1991).

Other critical factors for proper T cell activation include, but are not limited to, adhesion molecules. Adhesion molecules have been shown to enhance cell-cell interactions and have been hypothesized to provide costimulation for T cells (Clark and Ledbetter.1994; Damle *et al.*,1993). Also, the type of APC, the maturity of the T cell, the requirement for T cell help and the route, dose, form and persistence of antigen administration are clearly important in influencing T cell activation (Miller and Morahan.1992; Weiss.1993; Ucker *et al.*,1992; Webb *et al.*,1994; Luqman and Bottomly.1992; Schwartz.1990; Sloan-Lancaster *et al.*,1994; Kearney *et al.*,1994; Sloan-Lancaster *et al.*,1993; Mamalaki *et al.*,1993). If

mature T cells are stimulated, and the above factors are less than optimal, T cell tolerance may be induced. Several post-thymic T cell tolerance mechanisms have been hypothesized, including antigen desensitization through TCR blockade/modulation, suppressor cell regulation, veto cell regulation, functional nonresponsiveness or anergy, and the deletion of responding T cells through apoptosis (Schwartz.1993). Recent *in vivo* studies using bacterial superantigens provide evidence for both deletion and anergy induction as mechanisms leading to nonresponsiveness to *in vitro* restimulation (Kabelitz and Wesselborg.1992; Webb *et al.*,1994; Webb *et al.*,1990; Kawabe and Ochi.1991; MacDonald *et al.*,1991; Migita and Ochi.1993; Renno *et al.*,1995). Staphylococcal enterotoxin B activates a sub-population of T cells by binding the TCR Vβ8 chain and nonpolymorphic regions of MHC class II molecules on APCs (Marrack *et al.*,1990). This interaction leads to clonal expansion of TCR $V\beta8^+$ cells followed by a subsequent depletion of most of these cells from the periphery and induction of anergy in the remaining $V\beta8^+$ cells (MacDonald *et al.*,1991). The depletion of TCR $V\beta8^+$ cells was subsequently shown to be associated with DNA fragmentation, a hallmark of apoptosis (Kawabe and Ochi.1991). The anergy in thymectomized SEB-treated mice was reversible, but the recovery of the *in vitro* SEB response in treated mice was slow, requiring four months to equal that of untreated controls (Migita and Ochi.1993).

The mechanism of superantigen-induced AICD is not well understood. *In vitro* studies with human T cell lines revealed that the proliferative response to staphylococcal enterotoxin A (SEA) was independent of B7-dependent costimulation (Damle *et al.*,1993). In contrast, *in vivo* experiments with SEA provided data that demonstrate costimulation prevents T cell depletion. Co-treatment of mice with SEA and cognate antigen (pigeon

cytochrome C), both of which require Vβ3$^+$ and Vβ11$^+$ responder T cells, prevent SEA-induced deletion of these cells. These findings indicate a role for costimulation because cognate antigen activation, which induces B7-dependent costimulation, is able to prevent SEA-induced deletion of the Vβ3$^+$ and Vβ11 T cells (McCormack *et al.*, 1994). Conversely, another *in vivo* experiment provided data to suggest that costimulation did not affect the outcome of SEB-induced deletion of T cells. CD28 costimulation is known to increase the production of IL-2 but Vβ8$^+$ T cells were deleted equally well by SEB in the presence of the IL-2 synthesis inhibitor cyclosporin A or a recombinant vaccinia virus vector which constitutively produced large amounts of IL-2 (Gonzalo *et al.*, 1992). Taken together, these data may suggest that treatment with SEB and SEA are different. Administration of SEB leads primarily to T cell deletion and not anergy; IL-2 does not alter the outcome. Treatment with SEA may induce deletion and anergy; as co-treatment with cognate antigen (which is known to induce costimulation via IL-2 production) can divert T cells from both anergy and depletion. How SEB-responsive T cells choose between anergy and deletion *in vivo* is not clear, but recent findings suggest that if the responding T cell is sufficiently activated to proliferate it will undergo AICD while those T cells which do not proliferate become anergic. These results indicate a critical role for the initial strength of the TCR-derived signal one and/or the availability of costimulatory signals in determining the outcome of T cell activation, and, in the case of SEA administration, suggest that anergy might be a step toward AICD.

AICD is the least understood mechanism of T cell tolerance but is probably the most effective since it results in the specific deletion of targeted T cells. T lymphocytes treated

with anti-CD3 antibodies *in vitro* undergo AICD exhibiting characteristics of apoptosis. The term apoptosis was first described in 1972 and literally means "leaves falling" with inference to a natural process of death (Cohen *et al.*, 1992). Apoptosis describes a set of common morphological characteristics associated with a natural noninflammatory process of cell death. Well defined morphological and biochemical characteristics have been observed in the apoptosis of lymphocytes. These characteristics include cellular shrinkage, chromatin condensation and subsequent cleavage of DNA into oligonucleosome fragments, membrane blebbing and the loss of normal membrane symmetry with abnormal expression of phosphatidylserine on the outer leaflet of the plasma membrane (Schwartz and Osborne.1993; Squier *et al.*, 1995). DNA fragmentation appears to precede the other markers of apoptosis, and blocking DNA fragmentation with aurin tricarboxylic acid (ATA) in anti-CD3 stimulated splenic T cells and T cell hybridomas *in vitro* prevented apoptosis (Mogil *et al.*, 1994). Following *in vivo* induction of apoptosis, the cells remain viable until they are engulfed by phagocytes. *In vitro*, the cells eventually break apart into small membrane enclosed fragments which contain cytoplasmic organelles referred to as apoptotic bodies. The process of apoptotic cell death is not inflammatory, as is necrotic cell death, and occurs during normal physiological events such as embryogenesis and thymic T cell development and is believed to occur in lymphocytes as a result of activation (Gerschenson and Rotello.1992; Cohen *et al.*, 1992). Lymphocyte susceptibility to anti-CD3 antibody-induced apoptosis is associated with activation as demonstrated by *ex-vivo* studies showing apoptosis of antigen-sensitized but not nonsensitized transgenic splenocytes (Russell *et al.*, 1991). This differential response could be due to quantitative differences at the level of TCR signaling

or differences in the nature of costimulation. Similar experiments in *lpr* mice, which have a defect in the activation-associated *fas* antigen, showed a different outcome. T cells in these animals initially respond to anti-CD3 antibody stimulation, but, once activated, fail to undergo AICD (Kabelitz *et al.*, 1993). This suggests that the *fas* pathway is required for activation-induced apoptosis; the significance of this will be discussed later.

In vitro studies of transformed T cell lines and of antigen-activated nontransformed T cells have demonstrated the requirement for *de novo* transcription and translation in the apoptotic process (Ucker *et al.*, 1989). Other studies have shown that entry into the cell cycle is a requirement for AICD, since inhibitors of cell progression from G1 to S phase are able to prevent apoptosis (Zhu and Anasetti. 1995). Further studies on the dependence of cell cycle progression for AICD revealed that progression *per se* was not important, but cells had to be in the S phase of the cell cycle to be susceptible to anti-CD3 antibody-induced apoptosis. In these experiments nontransformed Th1 $CD4^+$ T cell lines and $CD8^+$ T cell lines were sensitized to cell cycle-dependent apoptosis when pretreated with IL-2 and IL-4, T cell cytokines known to drive T cells into the cell cycle (Boehme and Lenardo. 1993). The authors termed this phenomenon "propriocidal regulation", referring to a self-regulatory mechanism to prevent bystander T cell activation through the initiation of apoptosis. *In vitro* studies with anti-CD3 antibody reactivation of mitogen or alloantigen-activated murine splenic T cells revealed a requirement for at least two cell cycles in the presence, but not in the absence, of other splenic cell populations before apoptosis was induced (Radvanyi *et al.*, 1993). These data suggest that additional APC-derived signals are required for the prevention of AICD.

Alternatively, the strength of the TCR signal may by itself determine the outcome of T cell activation. T cell proliferation is inhibited in the presence of large doses of antigen or anti-TCR antibodies. This phenomenon, termed "high zone suppression" has been observed when T cells receive too strong of a TCR signal that subsequently prevents antigen-specific responses (Jenkins *et al.*, 1990; Miller and Morahan.1992; Critchfield *et al.*, 1994; Ferber *et al.*, 1994; Moskophidis *et al.*, 1993). A plausible hypothesis to explain these observations would propose T cell apoptosis to control T cells activated in the absence of costimulation or hyper-activated T cells in the presence or absence of costimulation as a mechanism to prevent autoimmunity or control T cell-mediated inflammation.

Cytokine signaling in T cells. Cytokines provide costimulatory signals and mediate T cell proliferation and differentiation. Broad-based immunotherapy with anti-CD3 antibodies in mammals has profound effects on transplant survival, but serious side effects often accompany treatment. These side effects were are the result of the systemic release of cytokines and the subsequent toxicities they generated. Previous investigators established a pattern of cytokine gene expression following *in vivo* administration with anti-CD3 antibodies, OKT3 in humans and 145-2C11 in mice. This pattern includes rapid expression of IL-2, IL-3, IL-4, IL-6, GM-CSF, INF-γ and TNF-α (Hirsch *et al.*, 1989; Flamand *et al.*, 1990; Alegre *et al.*, 1991; Ferren *et al.*, 1990; Scott *et al.*, 1990). Activation and/or proliferation signals in T cells may be mediated by IL-1, IL-2, IL-4, and IL-6 among others. Exposure of naive T cells to plate bound anti-CD3 antibodies *in vitro* results in the production of only IL-2 which suggests requirements other than cross-linking of the TCR to

induce the expression of most cytokines (Jenkins *et al.*, 1990). Anti-CD3 antibody-induced cytokines are produced by T cells *in vivo* in a tightly regulated manner which requires TCR cross-linking by FcγR-bearing cells. What is unclear, are the exact roles, if any, that the anti-CD3 antibody elicited cytokines may play in the induction of T cell tolerance. The majority of *in vivo* toxicity following anti-CD3 antibody treatment is caused by TNF-α, as evidenced by the ability of anti-TNF-α receptor antibodies to alleviate the toxicity normally observed following treatment. TNF-α is a potent inflammatory cytokine which has many functions, including the direct induction of apoptosis in certain cell types, but anti-TNF-α antibody treatment does not block the ability of anti-CD3 antibody treatment to inhibit graft rejection (Alegre *et al.*, 1990; Ferran *et al.*, 1991). This suggests that TNF-α induced apoptosis is not the cause of anti-CD3 antibody-mediated T cell depletion.

In vitro studies with Th1 and Th2 clones have shown a differential response to anti-CD3 antibody-induced anergy that is correlated to the cytokines produced. Exposure of Th1, but not Th2 clones, to anti-CD3 antibody leads to a block in both IL-2 production and proliferation upon restimulation with antigen and APC. This anergy was also seen in small resting T-cell cultures when incubated with anti-CD3 antibody and IL-2 but not anti-CD3 antibody and IL-4 (Gilbert *et al.*, 1990; Williams *et al.*, 1992). *In vitro*, Th1 cells require costimulation via CD28 to induce production and utilization of IL-2, which leads to proliferation. The costimulatory signal in Th2 clones can be provided through CD28, but IL-1 can substitute as a costimulatory molecule. The IL-1 costimulatory signaling allows the cells to respond to IL-4, produced in an autocrine fashion, which leads to proliferation (Lichtman *et al.*, 1988; Seder *et al.*, 1994; McArthur and Raulet.1993). Anti-CD3 antibody

treatment of Th1 helper T cells induces anergy, but these cells can be rescued through the addition of exogenous IL-2. Even though these cells initially produce low levels of IL-2, the basis of their anergic state is their failure to continue production of IL-2 (Jenkins *et al.*, 1990).

In addition to the role that IL-2 plays in the activation of T cells and the prevention of anergy, it also plays a role in AICD. $CD8^+$ CTL clones were shown to be sensitized to anti-CD3 and anti-Thy-1 antibody-induced apoptosis by co-treatment with IL-2. This response was dose-dependent on the concentrations of both the IL-2 and the anti-CD3 antibody (Ucker *et al.*, 1992). These results suggest that IL-2 may be important in preventing anti-CD3 antibody-induced apoptosis only if provided after T cell activation. The role of IL-2 *in vivo* was addressed through the creation of IL-2 knockout mice. These mice have reduced serum immunoglobulin and *in vitro* T cell responses, but *in vivo* they make an adequate antibody response to VSV and significant CTL response to LCMV (Schorle *et al.*, 1991; Kundig *et al.*, 1993). In addition, it was subsequently shown that if the mice were not kept in a germ-free facility they developed inflammatory bowel disease and eventually died. The inflammatory bowel disease was associated with a large number of activated B and T cells (Sadlack *et al.*, 1993). The high number of activated lymphocytes supports the hypothesis of "propriocidal regulation" of T cells mentioned above, since these cells may have been nonspecifically activated but not eliminated in the absence of IL-2. Taken together, these results suggest that the anti-CD3 antibody-induced anergic state may lead to apoptosis in the absence of IL-2. If IL-2 is provided before activation at a sufficiently high dose, T cells may be sensitized to undergo AICD as a mechanism to control bystander

activation. If IL-2 is provided during and after activation in the proper dose it may prevent AICD and lead to T cell activation and differentiation.

In vitro studies suggest a role for IL-2 in priming T cells for apoptosis when they are subsequently activated by anti-CD3 antibody or superantigens. However, a role for IL-2 *in vivo* is less clear. Following *in vivo* treatment with SEB, T cells undergo apoptosis when restimulated with anti-CD3 antibodies *in vitro*. This response can be partially inhibited by anti-IL-2R antibody but not anti-IL-4 antibody (Lenardo.1991). These findings are contrary to a subsequent finding in which SEB-induced apoptosis of TCR $V\beta8^-$ T cells *in vivo* occurred, both in the presence and absence of IL-2 which suggests that IL-2 does not play a role in SEB-induced apoptosis (Gonzalo *et al.*,1992). A separate *in vitro* study demonstrated anti-CD3 antibody-induced apoptosis of short term T cell clones but not freshly isolated resting PBMCs. Apoptosis of the T cell clones occurred in the presence of exogenous IL-2 and after 15-20 days in culture. The PBMCs also underwent anti-CD3 antibody-induced apoptosis, that was dependent on IL-2, indicating that IL-2 may be required for anti-CD3 antibody-induced apoptosis (Wesselborg *et al.*,1993).

Other cytokines may also play important roles after stimulation with anti-CD3 antibodies. It is possible that apoptosis is a regulatory response to the over-production of cytokines (*i.e.*, T cells produce cytokines in response to TCR cross-linking then commit suicide as a mechanism of regulating the response). It is also possible that there are distinct subsets of T cells *in vivo* that initiate apoptosis of other activated cytokine-producing T cells but are resistant to TCR-triggered apoptosis themselves. A unique $CD3^{low}$ $CD4^-$ $NK1.1^-$ cytokine-producing T cell was found in the murine thymus and bone marrow which appears

to be extrathymically derived. When this cell is stimulated with anti-TCR antibodies *in vitro*, it secretes comparable levels of IL-2 and higher levels of IL-4, IL-5, IL-10, and IFN-γ than single positive $CD4^+$ $NK1.1^-$ cells (Arase *et al.*, 1993). The $CD4^+NK1.1^+$ cells, but not $CD4^+$ $NK1.1^-$ cells, produced increased amounts of IL-4 mRNA when stimulated *in vivo* with anti-CD3 antibodies or the SEB superantigen. This increase in IL-4 mRNA was found to require B7-dependent costimulation since the fusion protein, CTLA4-Ig, was able to significantly inhibit the response. This cell population may represent a regulatory subset whose function may be to prime the immune response by producing cytokines which influence T cell activation and development of effector functions (Yoshimoto and Paul.1994).

The induction of IL-4 expression following T cell activation is a model for studying T cell activation pathways *in vivo*. Infection with the helminth, *Heligmosomoides polygyrus*, led to the development of a Th2 response resulting in the development of IL-4-producing $CD4^+$ T cells. The production of IL-4 during the primary response can be blocked with the use of the fusion protein CTLA4-Ig, suggesting that costimulation is required for antigen-responsive T cells that produce IL-4 *in vivo* (Lu *et al.*, 1995; Lu *et al.*, 1994). The requirement for costimulatory signaling in anti-CD3 antibody-induced cytokine gene expression *in vivo* is unclear.

The role of apoptosis-associated genes. Several recent studies have identified proteins involved in the initiation or prevention of apoptosis. APO-1/Fas is a cell surface protein that belongs to the TNF-α-R/NGF-R family. It is primarily expressed on cells with

high proliferative potential such as lymphocytes in the thymus, spleen and lymphatics but mRNA is also abundant in nonlymphoid cells of the liver, heart, kidneys, lungs and ovaries (Vignaux *et al.*,1995; Watanabe-Fukunaga *et al.*,1992; Nagata and Golstein.1995). Antibodies against this protein induced apoptosis in activated T cells which expressed *fas;* but did not induce apoptosis in naive T cells or T cells that did not express *fas* (Owen-Schaub *et al.*,1992). Anti-Fas antibody-mediated apoptosis of activated *fas*-expressing cells requires antibody cross-linking, since $F(ab')_2$ fragments were unable to induce apoptosis either *in vitro* or *in vivo* (Crispe.1994). Mice that have the lymphoproliferative disorder, *lpr,* were subsequently found to have a recessive mutation of the *fas* gene. The *lpr* mutation leads to transcriptional termination of most *fas* mRNA, but some full length transcripts are found, suggesting that some Fas protein may be produced (Watanabe-Fukunaga *et al.*,1992; Watanabe-Fukunaga *et al.*,1992). Cells from *lpr* mice are resistant to anergy induction and apoptosis following high dose anti-CD3 antibody treatment *in vitro* (Bossu *et al.*,1993). These findings were confirmed and expanded to show that there was no difference in the response to anti-CD3 antibody stimulation between effector cell populations in *lpr* mice. Th1 and Th2 T cells derived from *lpr* mice proliferated and produced IL-2 and IFN-γ or IL-4 in response to anti-CD3 antibody but failed to undergo apoptosis in contrast to wild type control T cells. The results suggest that *fas* signaling may be required for anti-CD3 induced apoptosis (Russell *et al.*,1993). In contrast to these results, T cells from *lpr* mice undergo rapid apoptosis when placed in culture without additional stimulation suggesting that *fas*-dependent signaling may not be the only mechanism of apoptosis induction (Van Houten and Budd.1992).

The ligation of Fas on human T cell lines results in the rapid phosphorylation of several cellular proteins prior to the initiation of apoptosis: the use of inhibitors of protein tyrosine kinase prevents apoptosis (Eischen *et al.*, 1994). The observation that PTK blockers inhibit Fas-induced apoptosis may explain the previous observation that anti-CD3 antibody stimulated apoptosis of thymic T cell cultures could also be blocked with PTK inhibitors (Migata *et al.*, 1994). These results may suggest that signaling through the TCR and/or Fas involves a separate or shared PTK activity which is required to initiate apoptosis.

The gene for Fas ligand *(fasL)* was recently cloned and sequenced and was found to be structurally related to TNF-α (Suda *et al.*, 1993). A point mutation in the C terminus of *fasL* which abolishes binding to *fas* was found to be the basis for the general lymphoproliferative disease *(gld)* phenotype. Mice homozygous for the *gld* mutation express a phenotype similar to *lpr* mice (Russell and Wang. 1993; Takahashi *et al.*, 1994). Expression of *fasL* appears to be restricted to activated lymphocytes, and cells expressing recombinant *gld fasL* could not induce apoptosis in *fas*-bearing target cells (Ramsdell *et al.*, 1994; Nagata and Golstein. 1995). Some evidence suggests that *lpr* mice and also *gld* mice may have a defect in peripheral T cell deletion but not thymic deletion (Singer and Abbas. 1994). *In vitro* studies with peripheral T cells from *lpr* and *gld* mice have demonstrated that there is a defect in anti-CD3 antibody-induced T cell apoptosis (Ohteki *et al.*, 1990; Reap *et al.*, 1995).

The *lpr* and *gld* mice accumulate large numbers of unusual lymphocytes: $CD4^-/CD8^-$ T cells, which are $TCR\alpha\beta^+$, $Thy1^+$ and also express the B cell marker B220. The accumulation of these aberrant lymphocytes is believed to be due to defects in either *fas* or *fasL*-dependent AICD. These lymphocytes do not accumulate until later in life, and the

accumulation of these cells can be repressed by neonatal thymectomy which suggests that the defect is primarily due to accumulation of thymus-derived T cells (Crispe.1994; Theofilopoulos *et al.*, 1981). It was also shown that the accumulation of these cells could be prevented by the addition of a *fas* transgene under the control of a T cell-specific CD2 promoter which suggests that the expression of *fas* prevents the accumulation of activated lymphocytes by elimination through the *fas*-dependent apoptotic pathway (Wu *et al.*, 1994).

The role of *fas* in antigen-specific apoptosis was studied in *lpr* mice with transgenic TCRs for a peptide of pigeon cytochrome C. Following administration of peptide, peripheral T cells in *lpr* mice, but not wild type mice, were resistant to peripheral T cell deletion. In contrast, thymocytes in both backgrounds were susceptible to peptide-induced deletion which suggests that activation-induced deletion of peripheral T cells, but not thymocytes, is dependent on *fas*-mediated apoptosis (Singer and Abbas.1994). Although *fas*-dependent apoptosis was suggested as the mechanism of T cell deletion, no direct evidence for apoptosis was provided. Additional evidence for *fas*-mediated activation-dependent apoptosis was generated in three separate studies using human T cell lines and murine hybridoma cells in limiting dilution analysis (Dhein *et al.*, 1995; Brunner *et al.*, 1995; Ju *et al.*, 1995). These *in vitro* studies all demonstrated that anti-CD3 antibody or SEB-induced T cell apoptosis occurs through enhanced *fas* and *fasL* expression. This apoptosis required *fas*-mediated signaling since anti-fas blocking antibodies or chimeric *fas*-Fc constructs were able to abolish AICD of cultured single cells. Taken together, these data suggest that activation of T cells through the TCR induces up regulation of *fas* and the subsequent *fas*

signaling may induce a cellular suicide process which *in vitro* is mediated by the autocrine production of soluble *fasL*.

A final set of genes which may play an important regulatory role in apoptosis include *bcl-2* and the related *bax* genes. *bcl-2* was originally described as the over expressed protein responsible for a B cell lymphoma, suggesting that it played a role in the activation and maintenance of lymphocytes (Tsujimoto and Croce.1986). *bcl-2* is expressed in $CD4^+$ or $CD8^+$ single positive but not $CD4^+$, $CD8^+$ double positive thymocytes. It is also expressed in resting peripheral T cells suggesting that its presence may mediate cell survival and its absence may facilitate negative selection in the thymus through AICD (Veis *et al.*, 1993). *bcl-2* was also expressed in the majority of murine thymic single-positive cells and peripheral memory T cells but not double-positive thymocytes. *bcl-2* expression by memory T cells is down-regulated following their activation. These data suggest that *bcl-2* may play a role in the prevention of apoptosis of mature thymic T cells or in the maintenance of peripheral T cells and possibly immunological memory (Veis *et al.*, 1993; Akbar *et al.*, 1993).

The *bcl-2* protein is associated with the mitochondrial and nuclear membranes and suppresses apoptosis following growth-factor withdrawal in the murine IL-3 dependent cell line FDC-P1. Over expression of *bcl-2* in this cell line partially inhibited *fas*-dependent apoptosis (Itoh *et al.*, 1993). *bcl-2* knockout mice have mature lymphocytes for the first four weeks of life which then disappear from the thymus, bone marrow and the periphery. *In vitro*, mature T cells from these mice had increased sensitivity to apoptosis induced by gamma-radiation and glucocorticoid treatment as well as decreased sensitivity to anti-CD3 antibody stimulation (Veis *et al.*, 1992). These data suggest that *bcl-2* may play a role in

balancing T cell activation and cell death, possibly representing a homeostatic control mechanism. The interplay of the *fas*, *fasL* and *bcl-2* genes may control the fate of activated T cells.

Purpose of the study. The purpose of this study is to examine the regulatory mechanism of the *in vivo* immune response that discriminates between peripheral T cell activation leading to either cytokine production or apoptosis by using a mitogenic antibody against the invariant CD3ϵ chain of the TCR-complex. Preliminary studies focused on the development of *in vivo* assays to detect apoptosis and were followed by studies on the requirements for T cell activation leading to cytokine production *vs.* AICD. It is hypothesized that: 1) anti-CD3 antibody treatment induces AICD by the mechanism of apoptosis, 2) anti-CD3 antibody-induced apoptosis and cytokine production require endogenous B7-dependent costimulation and 3) anti-CD3 antibody-induced apoptosis requires the *fas/fasL* signaling pathway. These studies should provide insights about the function of the anti-CD3 antibody *in vivo* and should provide a useful model relevant to understanding T cell reactivity following TCR-mediated signal transduction in the presence or absence of costimulatory signals.

MATERIALS AND METHODS

Mice. Six to eight week old female mice were used for all experiments except where specified. BALB/c and C57BL/6 mice were obtained from NCI, NIH (Frederick, MD). B6.*lpr* and age matched C57BL/6J mice and C3H/OuJ mice were obtained from Jackson Laboratories (Bar Harbor, Maine). B6.μ*MT* mice were a kind gift of Dr. Fred Finkelman and Dr. Jimmy Mond, Department of Medicine, USUHS. All mice were housed in the USUHS laboratory animal facility and were fed standard lab chow and water *ad libitum*. *In vivo* experiments were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, Department of Health, Education and Welfare (NIH) 78-23.

Reagents.

Antibodies for injection. Hamster anti-mouse anti-CD3 antibody 145-2C11 and isotype control hamster IgG were generous gifts of Dr. Fred Finkelman, Department of Medicine, USUHS. Murine CTLA4-Ig and the control fusion protein L6 were the generous gifts of Dr. Peter Linsley, Bristol-Meyers Squibb, Seattle, WA. Hamster IgG anti-CD28 (37.51), was purchased from PharMingen (SanDiego, CA). Rat IgG2a anti-IL-2 (S4B6) and rat IgG1 anti-IL-2R (PC-61) and control isotype antibodies GL117 (IgG2a) and GL113 (IgG1) were also generous gifts of Dr. Fred Finkelman, Department of Medicine, USUHS. All antibodies for injection were diluted to a concentration of 1μg/μl in sterile PBS (Quality Biological, Inc. Gaithersburg, MD) and stored at -70°C.

Cytokines/cytokine complexes for injection. IL-2 complexes consisting of recombinant human IL-2 and anti-human IL-2 antibody (5B1) and IL-4 complexes consisting of recombinant murine IL-4 and anti-murine IL-4 antibody (BVD4-1D11.2) were all generous gifts of Dr. Fred Finkelman, Department of Medicine, USUHS. Recombinant human IL-1α was a generous gift of Dr. Ruth Neta, Department of Hematology, AFRRI.

Antibodies for cell staining. Antibodies used for cell surface labeling are included in Table 1. Antibodies were diluted in HBSS with 0.1% BSA and 0.1% sodium azide or HBSS with 1.0 % newborn calf serum and 0.2 % sodium azide. The working stocks were stored at 4°C. The amount of each antibody used for labeling $2x10^6$ cells was determined empirically.

Table 1. Antibodies for Cell Surface Labeling.

Antibody	ISO/ALLOtype	Origin	Target	Specificity
11B11	IgG1	Rat	Mouse	IL-4
2.43	IgG2b	Rat	Mouse	Lyt2/CD8
24G2	IgG2b	Rat	Mouse	FcR
145-2C11	IgG	Hamster	Mouse	CD3ϵ
6B2	IgG2a	Rat	Mouse	B220/CD45R
7D4	IgM	Mouse	Mouse	IL-2R
CBPC-101	$IgG2a^b$	Mouse	Mouse	
DS-1	$IgG1^b$	Mouse	Mouse	IgM^a
GK1.5	IgG2b	Rat	Mouse	L3T4/CD4
GL113	IgG1	Rat	Mouse	β-Gal
GL117.41	IgG2a	Rat	Mouse	β-Gal
HamIgG	IgG	Hamster		
MB86	$IgG1^a$	Mouse	Mouse	IgM^b
MKD6	IgG2a	Mouse	Mouse	Ia^d
PK136	IgG2a	Mouse	Mouse	NK1.1
PK136*	IgG2a	Mouse	Mouse	NK1.1
53-2.1*	IgG2a	Rat	Mouse	Thy1.2/CD90
3C7*	IgG2b	Rat	Mouse	IL-2R/CD25
37.51*	IgG	Hamster	Mouse	CD28
H57-597*	IgG	Hamster	Mouse	TCRαβ
145-2C11*	IgG	Hamster	Mouse	CD3ϵ
Jo2*	IgG	Hamster	Mouse	FAS

*Indicates antibodies purchased through ParMingen (San Diego, CA). All other antibodies were the generous gift of Dr. Fred Finkelman, Department of Medicine, USUHS.

Gene Expression.

RNA isolation. RNase-free plastic ware and water were used throughout. Tissues were homogenized in RNAzol B™ from Cinna Scientific, Inc. (Friendswood, TX) at 50 mg of tissue/ml RNAzol then were snap frozen in liquid nitrogen. Frozen homogenates were thawed for approximately five minutes in a 37°C water bath. RNA was extracted by the addition of a 24:1 mixture of chloroform/isoamyl alcohol at 0.2 ml for every 2 ml of homogenate. Samples were vortexed for 15 seconds and incubated on ice for five minutes. The samples were centrifuged for 15 minutes at (4°C) at 12,000 x *g*. The aqueous (top) phase, containing RNA, was transferred to another tube, and placed on ice. RNA was precipitated by addition of a volume of cold isopropanol which is equal to the volume of the aqueous phase. Tubes were mixed gently and stored for 15 minutes on ice. Samples were centrifuged for 15 minutes at 4°C, 12,000 x *g* to pellet the RNA. The RNA was washed by decanting the isopropanol and adding a volume of cold 75% ethanol and resuspending the RNA pellet by gently shaking or pipetting. The sample was centrifuged for eight minutes at 4°C, 12,000 x *g*. The ethanol was decanted and the pellets were dried by air or under vacuum. RNA pellets were solubilized in 20-50 µl of distilled, deionized water and quantified spectrophotometrically by measuring the OD 260/280 of an aliquot.

Reverse transcription of RNA. The reverse transcriptase (RT) reaction described below is based on a starting concentration of 3.6 µg of total RNA in a 25 µl volume containing: 1) 2.5 µl Deoxynucleotide Triphosphates (dNTPs) (Pharmacia, San Diego,CA) 2.5 mM each dNTPs. 2) 2.0 µl 0.1M Dithiothreitol (GIBCO BRL, Gaithersburg, MD). 3)

0.5 µl RNAsin (40,000 U/ml) (Promega, Madison, WI). 4) 2.0 µl Random Primers (N)6 (20-40 U/ml) (Boehringer Manheim, Indianapolis, IN). 5) 11.8 µl RNA (diluted with distilled/deionized H_2O). The above mixture was then heated to 70°C for five minutes and quenched on ice. To each sample 6.2 µl of GIBCO BRL Reverse Transcriptase Buffer (5.0 µl) and GIBCO BRL Reverse Transcriptase 200 U/µl (1.2 µl) was added. The mixture was incubated at 37°C for 60 minutes followed by denaturing at 90 C for five minutes and quenching on ice for five minutes. Samples were stored at -70°C.

PCR. The primers for mRNA amplification are listed in Table 2. These primers were designed as previously described (Gause and Adamovicz.1994) with the addition of a requirement to amplify genomic introns to discriminate between amplified cDNA and genomic DNA. The reaction described below is based on using 2.5 µl of R.T. reaction product (cDNA) with components in the following concentrations: 1) 4.0 µl Deoxynucleotide Triphosphates (Pharmacia, 2.5 mM each dNTPs). 2) 5.0 µl Promega Taq DNA Polymerase Buffer A (10X). 3) 3.0 µl $MgCl_2$ (Promega, 25 mM). 4) 0.2 µl Taq DNA Polymerase (Promega, 5 U/µl). 5) 2.0 µl sense oligo primer (0.2 µg/µl). 6) 2.0 µl antisense oligo primer (0.2 µg/µl). 7) 31.3 µl distilled, deionized water. The mixture was overlaid with 50 µl of mineral oil. After an initial denaturing at 94°C for five minutes the mixture was temperature cycled as follows: 1) Denaturing 94°C for 45 seconds. 2) Annealing 53°C for one minute. 3) Extension 72°C for two minutes; with a final extension cycle at 72°C for seven minutes. The samples were stored at 4°C. For each gene product the optimum number of cycles were determined experimentally and defined as the number of

cycles that would detect two fold increases in input RNA in a linear range. To normalize for the differences of starting RNA between samples, primers for the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) were used to amplify an aliquot of cDNA from each sample tested. Since HPRT expression rarely varies more than two to three fold, it's expression can be used to control for differences in the starting mRNA concentration between samples, and thus normalizes the resulting values for cytokine gene expression (Svetic *et al.*, 1991).

Table 2: Primer sequences for amplification of cDNA during PCR and probe sequences for detection of amplified product.

Gene	Anti-sense and Sense Primers	Bases^ Spanned	Probe	Bases Spanned	PCR Cycles	Ref
HPRT	GTTGGATACAGGCCAGACTTTGTTG GATTCAACTTGCGCTCATCTTAGGC	514-538 652-678 (164)	GTTGTTGGATATGCCCTTGAC	562-582	13	a
FAS	TGGTCAACAACCATAGGCG CTCAAGGTACTAATAGCATCTCCG	554-572 111-134 (461)	GCACAGAAGGGAAGGAGTAC	291-310	21	b
FAS-L	CACGAAGTACAACCCAGTTTCG CAACACAAATCTGTGGCTACCG	546-567 227-247 (340)	CCGTGAGTTCACCAACCAAAGC		26	c
BCL-2	GCGACCTCTGTTTGATTTCTCC GGTCATGTGTGTGGAGAGCG	3348-3369 2276-2295 (173)	GTACCTGAACCGGCATCTGC	2349-2369	22	d
TNF-α	TTCTGTCTACTGAACTTCGGGG ACTTGGGCAGATTGACCTCAGC	955-976 2437-2458 (514)	CCCGACTACGTGCTCCTCACC	2248-2268	23	e

NK1.1	GAAGCACAGCTCTCAGGAGTCAC CTACCTCGGTTTAAAGCCACC	24-44 577-599 (575)	GTTTCTCAAGTTTCCAACACTTG	310-332	22	f
IL-2	GAGTCAAATCCAGAACATGCC TCCACTTCAAGCTCTACAG	122-140 349-369 (247)	CTCCCCAGGATGCTCACCTTC	256-277	26	a
IL-4	GAATGTACCAGGAGCCATATC CTCAGTACTACGAGTAATCCA	110-130 472-493 (383)	AGGGCTTCCAAGGTGCTTCGCA	271-298	23	a
IL-10	CGGGAAGACAATAACTG CATTTCCGATAAGGCTTGG	147-163 315-333 (186)	GGACTGCCTTCAGCCAGGTGAA GACTTT	209-227	20	a
IFN-γ	AACGCTACACACTGCATCTTGG GACTTCAAAGAGTCTGAGG	73-94 291-310 (237)	GGAGGAACTGGCAAAAGGA	229-248	20	a

[A] Numbers indicate the base sequence positions that are bound by the anti-sense and sense primers respectively. The size of the expected PCR product is indicated inside parenthesis.

References: a. (Svetic *et al.*, 1991). b. (Watanabe-Fukunaga *et al.*, 1992). c. (Suda *et al.*, 1993). d. (Negrini *et al.*, 1987). e. (Shirai *et al.*, 1988).

Detection of PCR product by Southern blot analysis. A mixture of 9 µl of PCR product and 3 µl loading buffer were placed on a 300 ml 1% agarose TBE gel (100 mM Tris Base,10 mM EDTA, 2 M NaCl pH 7.4) and electrophoresed for 30-40 minutes at 120 V to separate primers and nonspecific amplification products from the amplified cDNA of interest. The gel was denatured by gentle shaking in a 1X alkaline solution (1.5 M NaCl, 0.5 M NaOH, pH 13) for 50 minutes with two volumes of solution. Next, the gel was rinsed in deionized water and neutralized for 30 minutes with two volumes of a 1X tris buffer solution (1.5 M NaCl, 1 M Tris-HCL pH 7.5). The gel was rinsed and saturated with salt in a 20X SSPE solution (3 M NaCL, 0.2 M $NaH_2PO_4H_2O$, 0.02 M EDTA, 0.213 M NaOH, pH 7.4). The PCR product was transferred by capillary action to a 0.2 µm nytran membrane, (maximum strength plus-positively charged nylon membrane, Schleicher and Schuell, Keene, NH), overnight. Subsequent immobilization of the DNA to the membrane was accomplished with 1,200 Joules of ultraviolet (UV) light cross-linking with a UV cross-linker (Stratalinker 1800, Stratagene, La Jolla, CA). Immobilized blots were prehybridized at 42°C for five h in a 15 ml solution containing : 6X SSPE, 10X Denhardts, 1% SDS and salmon sperm DNA (S.S.DNA) at a concentration of 50 µg/ml. The S.S.DNA was denatured by heating the required amount of S.S.DNA (about 25-50 µg/ml of solution) to 94°C for five minutes and then quenched on ice. Blots were hybridized at 49°C with ^{32}P labeled probes (Table 1) in 15 ml hybridization solution containing 6X SSPE and 1% SDS for 12-24 h. Labeling of oligonucleotide probes was accomplished in the following solution: 1) 2.5 µl Probe buffer (5 µl 1 M Tris pH 7.6, 0.5 µl 2 M $MgCL_2$, 1 µl 0.5 M Dithiothreitol, 3.5 µl Distilled water). 2) 2.0 µl Oligonucleotide (0.2 µg/µl). 3) 9.5 µl Distilled water. 4) 1.0 µl T4 Kinase

(Pharmacia). 5) 10.0 µl ^{32}P-ATP (10 µCi/µl) incubated at 37°C for 40 minutes. After the incubation was complete the non-incorporated label was separated from the labeled probe with a G-25 Sephadex spin column (5 prime-3 prime, Boulder, CO). The specific activity of each probe was determined using a liquid scintillation counter. Probes were designed to hybridize to a portion of the PCR product that did not overlap with sequences used for the amplification. The blots were washed by shaking for 15 minutes at 49°C in 6X SSPE, 0.1% SDS then for 30 seconds in 2X SSPE. After washing the blot the specific signal remaining was quantitated using a PhosphorImager™ (Molecular Dynamics, Sunnvale, CA). This technique involved exposure of the blot to a Phosphor screen which was then scanned by a PhosphorImager and analyzed by ImageQuant™ software (Molecular Dynamics, Sunnvale, CA). The specific values for gene expression were calculated by subtracting the background signal from the specific signal and then normalizing the expression value to the HPRT expression value from the same sample.

Flow Cytometry. Cell suspensions were prepared from the tissues of euthanized mice by pressing the organ through a fine wire mesh. For most experiments the cells were suspended in Hanks Balanced Salt Solution (HBSS) (BioWhittaker, Walkersville, MD) with 0.1% BSA and 0.1% sodium azide (Sigma, St. Louis, MO). After concentrating the cells by centrifugation at 1,100 x *g* for five minutes and decanting the supernatant, the RBCs were lysed in ACK lysis buffer (0.155 M Ammonium Chloride, 10 mM EDTA and 0.1 mM Potassium Bicarbanate, pH 7.4) for twenty seconds. Cells were then washed twice in HBSS. For experiments requiring incubation in media the RBC lysis was omitted. Concentrations

of the resulting cell suspensions were determined with a Coulter Model ZB1 counter (Coulter, Miami, FL). $2x10^6$ cells were concentrated in a volume of approximately 50 µl. Fc receptors were blocked with an anti-Fc receptor (FcR) antibody (24G2) to prevent nonspecific binding of antibodies. Cell staining was accomplished by the addition of antibodies (Table 1) and incubation of the cells on ice for 30 minutes. The cells were then washed in 1 ml of HBSS buffer and centrifuged at 1,100 x *g* for five minutes; the buffer was decanted and the cell pellet was gently disrupted in the tube. If a biotinylated primary antibody was used the cells were incubated with a streptavidin (SA) conjugate. The amount of conjugate used was pre-determined empirically. The conjugates used include: SA-phycoerythrin (PE) and SA-fluorescein (FITC) (Becton Dickenson, San Jose, CA). SA-allophycocyanin (APC), SA-CY-Chrome™ and SA-PE (PharMingen, San Diego, CA) and SA-7-amino-4-methylcoumarin-3-acetic acid (AMCA), (Molecular Probes, Eugene, OR). After staining, the cells were washed twice, resuspended in 0.5-1.0 ml of buffer, and filtered through nylon mesh. Single, and two color flow cytometry was performed on freshly stained cells using a FACS analyzer and Consort 30 software (Becton Dickinson, Sunnyvale CA). Two, three, and four color analyses were performed on a Coulter EPICS Elite using Workstation 4.01 software (Coulter, Miami, FL). Excitation of FITC, CyChrome™ and PE was accomplished with an argon laser at 488 nm and the resulting signals were measured through a 550 nm longpass dichroic filter at 525 nm, 675 nm and 575 nm respectively. SSC was detected by excitation at 488 nm with an argon laser and the signal was split from the fluorescent signals with a 488 nm bandpass filter. FSC signal was detected through a neutral density filter and 633 nm laser blocking filter. Three color experiments with Cy5 instead of

CyChrome™ were completed as above except the excitation of Cy5 was accomplished with a helium-neon laser at 633 nm offset by 20 μs from the primary argon laser excitation. Four color experiments with FITC, Cy5, PE and AMCA were completed by excitation of FITC and PE with an argon laser at 488 nm, Cy5 at 633 nm with a helium-neon laser offset from argon excitation by 20 μs and AMCA excitation at 325 nm with a helium-cadmium laser. Emission was detected by passage through 488 nm, 550 nm and 625 nm longpass dichroic filters with AMCA measured at 460 nm, FITC at 525 nm, PE at 575 nm and Cy5 at 675 nm. During the analysis red blood cells and debris were excluded on the basis of forward and 90° light scatter. Analysis gates were established based on unstained or single stained control profiles. Cell sorting was performed on the Coulter EPICS Elite and the collected cells were reanalyzed to determine population purity.

Protocols for *in vivo* studies.

The loss of T cells or T cell depletion was used as a parameter to measure T cell survival four days after treatment with 20-30 μg of anti-CD3 antibody. The depletion was measured by determining the percent of $CD4^+$ and $CD8^+$ T cells in the spleen or lymph nodes (brachial and axillary pairs). The number of cells was calculated by Coulter counting a sample of single cell preparations from the spleen or lymph nodes then multiplying the calculated total cell population number by the percent of those cells which were $CD4^+$ or $CD8^+$ as determined by FACS analysis described above.

Administration of exogenous cytokines IL-2, IL-4 or IL-1. BALB/c mice, three per group, were given lateral tail vein injections of exogenous IL-2/anti-IL-2 complexes (5 μg/25 μg) per dose, respectively at two time points. The first protocol involved the coadministration of one dose of IL-2 complex with either 30 μg anti-CD3 or hamster IgG antibody in a final volume of 300 μl sterile PBS containing 1% mouse serum followed by a second dose of IL-2 complex two days after the first. The second protocol employed the administration of IL-2 complexes 24 and 72 h following treatment. The animals were euthanized on day four and thymic and peripheral CD4 and CD8 cells in both the spleen and lymph nodes were enumerated. Mice in protocol two that were given both IL-2 complexes and anti-CD3 antibody died within 24 h of the first administration of IL-2 complex. Therefore, a second experiment included only a single injection of IL-2 complex on day zero or day one. Rescue of peripheral T cells from anti-CD3 antibody-induced deletion was scored by comparing the mean ± SEM cell number of CD4 or CD8 cells to animals given anti-CD3 antibody only. Exogenous IL-4 complexes (5 μg/25 μg) were given in a single dose six h before anti-CD3 or hamster IgG antibody and surviving peripheral CD4/CD8 cells were enumerated in two separate experiments as described above. Exogenous IL-1α was given in one or two μg doses one day before anti-CD3 or hamster IgG antibody treatment in three separate experiments and surviving peripheral CD4/CD8 cells were enumerated as described.

Addition of anti-IL-2/anti-IL-2R antibodies. Three-five BALB/c mice per group were given 1.5 or 3 mg each of both anti-IL2 and anti-IL-2R antibodies 12 h prior to 20 μg anti-

CD3 or hamster IgG antibody. Peripheral T cell depletion at day four was assessed. This experiment was performed three times at each dose.

Addition of exogenous LPS. Purified *E.coli* K235 LPS (1 μg/μl) was a kind gift of Dr. Stepenie Vogel, Department of Microbiology, USUHS. In several different dose-response experiments BALB/c mice, three per group, were given lateral tail vein injections in sterile PBS of 25, 12.5, 6, 4, 1, 0.5 or 0.25 μg of LPS either 24 h, 6 h, 1 h before or simultaneously with or 1 h, 6 h, 12 h or 18 h after 20 or 30 μg of anti-CD3 or hamster IgG antibody. Rescue of peripheral T cells from anti-CD3 antibody-induced deletion was determined by comparing the mean ± SEM number of $CD4^+$ or $CD8^+$ cells from mice given LPS and anti-CD3 antibody to the mice given anti-CD3 antibody only. In a different experiment, C3H/OuJ (LPS-responsive) mice, were given 1, 4 or 12 μg of LPS 12 h after anti-CD3 or hamster IgG antibody treatment. The 12 μg dose of LPS was lethal to anti-CD3 antibody treated C3H/OuJ mice..

Addition of exogenous CTLA4-Ig. The fusion protein CTLA4-Ig or the control fusion protein L6 were given to mice in two separate injections of 100 μg each, the first 24 h prior to treatment and the second simultaneously with hamster IgG or anti-CD3 antibody treatment unless otherwise noted. This dose inhibits the primary immune response to the helminth Heligmosomoides polygyrus (Lu *et al.*, 1994).

Protocols for *ex-vivo* studies.

DNA fragmentation. Detection of oligonucleosomal fragments of DNA, a hallmark of apoptosis was accomplished by a modification of a previously described technique (Perandones *et al.*, 1993). Preparations of $2x10^6$ sorted $Thy1^+$ cells or unsorted cells from anti-CD3 or hamster IgG antibody treated mice were lysed in hypotonic buffer (10 mM Tris, 1 mM EDTA, 0.2% Triton-X-100 pH 8). The samples were then ultracentrifuged 13,800 x *g* for 15 minutes and the supernatant (containing small DNA fragments) collected and mixed with an equal volume of isopropanol and 0.5 M NaCL. The nucleic acid was precipitated overnight at -20°C and the following day the samples were ultracentrifuged and the pellets washed in 70% ethanol. The pellets were air dried and resuspended in 20 µl of TE (10 mM Tris, 1 mM EDTA pH 7.4) and electrophoresed on a 1% TBE gel containing ethidium bromide (10 mg/ml) at 60 V for two h. The gel was then illuminated with UV light and photographed. Lanes containing apoptotic cell DNA have a characteristic banding pattern with multiples of 180-200 bp oligonucleosomal DNA fragments.

Fluorescent microscopy. Cells were examined for morphological characteristics associated with apoptosis as previously described with minor modifications (Coligan, 1991). Preparations of $1-2x10^6$ Thy1 sorted cells from anti-CD3 or hamster IgG antibody-treated mice were concentrated to a final volume of 20 µl and stained with a 2 µl mixture of acridine orange (AO) and ethidium bromide (EB), (0.1 mg of each in 100 ml PBS). An 8 µl aliquot of the mixture was placed on a coded 1 mm slide and covered with a No. 1, 22 mm^2 coverslip. Cells were counted on an Olympus BH-2 microscope (Tokyo, Japan) with UV

excitation. The fluorescent signal from AO stained viable cells was visible through a O530 contrast filter as green while the EB stained non-viable cells appeared orange. Sorted cells were scored for apoptotic morphology and counted at 400 X magnification under oil. Both viable and non-viable cells which had condensed, densely staining chromatin and/or a disrupted nuclear structure as evidenced by smaller apoptotic bodies, were counted as apoptotic cells. Photographs of cells were taken with an Olympus exposure control unit and 35 mm camera using AGRA 1000RS and 400RS film. Exposures were taken at both 400X and 1000X magnification under oil.

Cytokine production. The frequency of IL-4 producing cells was determined by an ELISPOT assay as previously described (Lu *et al.*, 1994). This assay is briefly described as follows: wells of an Immulon II plate (PGC Scientific, Gaithersburg, MD) were coated with anti-IL-4 antibody BV41D122 at 10 mg/ml and incubated overnight at 4°C. The plates were then washed three times in PBS Tween 20 (0.05%) followed by three washes in PBS, the plates were then blocked with RPMI 1640 and 5% FCS (BioWhittaker, Walkersville, MD) for one h at 37°C. One tenth milliliter aliquots of $5x10^6$ cells/ml were added to the coated plates in serial five-fold dilution and incubated for three h at 37°C. Plates were then washed three times with PBS followed by PBS-Tween 20. The biotinylated anti-IL-4 antibody BVD6.24G2 (4 μg /ml) was then added to the wells in 100 μl aliquots and the plates were incubated for one h at 37°C. The plates were washed three times in PBS and three times in PBS-Tween 20. Next, streptavidin-alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA), diluted 1/2000 in PBS-Tween 20 and 5% FCS, was added to the wells and the

plates were incubated for one h at 37°C. Plates were then washed five times in PBS and a solution of 0.6% LMP agarose containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate in 0.15 M 2-amino-2-methyl-1-propanol (Sigma, St. Louis, MO) was added to each well. After overnight incubation at room temperature in a humid chamber the number of blue spots in each well was counted with the aid of a dissecting microscope. Each spot represented an IL-4 secreting cell. Wells to which no cells were added served as negative controls and the mean number of spots/well was subtracted from all other experimental values.

FLANUL assay (FACS Labeling And Nick translation dUTP Labeling). This technique is a novel modification of the TUNEL assay (TdT-mediated dUTP-biotin Nick End Labeling) (Gavrieli *et al.*, 1992; Gorczyca *et al.*, 1993) and a protocol from Dr. Leslie King, NIH which had been used *in situ* to detect free 3' ends of DNA, a characteristic of cells undergoing apoptosis. This assay combines labeling of cell surface markers (immunotyping) and internal labeling of DNA by nick translation to identify subsets of cells with fragmented DNA. Single cell suspensions were prepared in DMEM-10 modified media containing 25 mM HEPES buffer (BioWhittaker, Walkersville, MD), 50 μM 2-mercaptoethanol, 2 mM L-glutamine and 100 μM nonessential amino acids (Gibco-BRL, Grand Island, NY) and the cells were incubated for two to three h in 6 or 24 well plates at 37°C in 6% CO_2. The surface of cells were labeled as previously described for FACS analysis, then fixed in 2.5% formaldehyde overnight at 4°C. The cells were washed twice in HBSS and permeated with methanol (-70°C) for two minutes. After permeation, the cells were washed twice with HBSS and incubated for two h at 37°C in a 50 μl solution containing the following reagents:

1) 5 µl of 10X Buffer (500 mM Tris, 100 mM $MgSO_4$, 1 mM DTT, pH 7.5). 2) 3 µl (0.01 nMol/µL) FITC labeled or biotin conjugated dUTP (Boehringer-Manheim, Indianapolis, IN). 3) 1 µl of each dNTPs (0.7 nM/µl dTTP, 1 nM/µl dATP, dCTP, dGTP). 4) 1 µl DNA polymerase I (Boehringer-Manheim, Indianapolis, IN). 5) 40 µl of distilled-deionized water. Following incubation cells were throughly washed in modified HBSS and if required they were further incubated with a streptavidin conjugate. Multi-parameter fluorometric analysis was used to identify apoptosing cell populations on the EPICS Elite as described above. Analysis quadrants for the UTP signal were established with control samples to which all reagents were added except DNA polymerase.

Statistical analysis. The software SAS version 6.10 was used for all statistical analysis. General linear models-(PROC GLM), were used for all group comparisons. Experiments which involved more than two treatments or multiple factors were examined by the (PROC TTEST)-analysis of variance, followed by designed pairwise contrasts of treatments (SAS/STAT Users Guide, 1989).

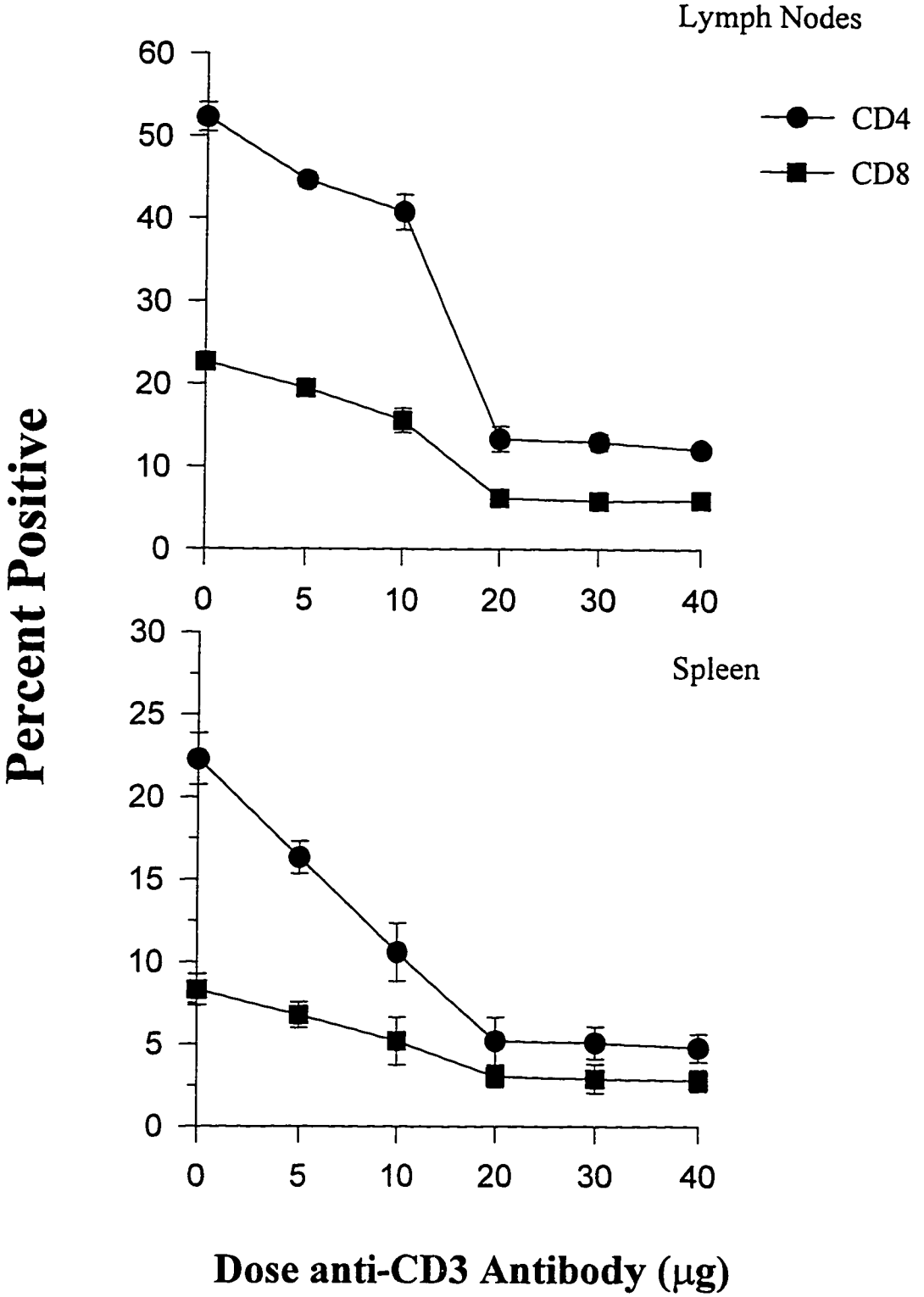
Results

Part I. *In vivo* administration of anti-CD3 antibody induces apoptosis of peripheral T cells.

Loss of T cells from peripheral lymphoid organs following in vivo treatment with anti-CD3 antibody is dose-dependent. Previous studies have indicated that T cell tolerance following iv injection with anti-CD3 antibody is due to T cell deletion (Hirsch *et al.*, 1989). These studies relied on large doses (400 μg) of anti-CD3 antibody which produced complications attributed to the release of inflammatory cytokines including: hypothermia, ischemia, tubular necrosis, hypoglycemia, and in some cases, death. To determine whether lower, less toxic anti-CD3 antibody doses could still cause T cell loss, dose-response experiments with anti-CD3 antibody were performed and changes in the number of splenic and lymph node T cells were measured four days after treatment. The use of 50 μg of anti-CD3 antibody had been shown to reduce splenic $CD4^+$ cell counts by greater than 90% in eight week old BALB/c mice four days following administration (Dorothy Scott, NIH, personal communication). Mice were given 5-40 μg of anti-CD3 antibody and four days later were euthanized; the remaining splenic $CD4^+$ and $CD8^+$ cells were enumerated (Figure 1). The observed T cell deletion was dose-dependent and the dose of 20-30 μg dose of anti-CD3 antibody was chosen for future experiments because it consistently reduced the splenic and lymph node T cell counts by greater than 75% four days after treatment.

Figure 1. Dose-dependent loss of T cells from peripheral lymphoid tissue. BALB/c mice were given iv injections of various doses (μg) of anti-CD3 antibody in sterile PBS and were euthanized four days later. Brachial and axillary lymph node pairs and whole spleen cells were prepared and counted on a Coulter counter. $2x10^6$ cells from each tissue were labeled with antibodies against the cell surface markers CD4 and CD8. The percent of CD4 or CD8 cells was calculated using a live gate analysis of cells multiplied by the total number of cells from the tissue. The mean (n=3) ± SEM for the number of recovered $CD4^+$ or $CD8^+$ positive lymphocytes is displayed, these data are representative of two independent experiments.

T cell Deletion After Anti-CD3 Antibody Treatment



Kinetics of peripheral T cell deletion in both the spleen and lymph nodes following in vivo treatment with anti-CD3 antibody. To determine the time course of T cell deletion following anti-CD3 antibody administration mice were given 20 μg doses of anti-CD3 or control hamster IgG antibody four, three, two, one day or 12 h before euthanasia. The remaining peripheral T cells; splenic or lymph node preparations, were enumerated by Coulter counting and then stained with antibodies to the T cell surface markers CD4 and CD8 and analyzed by FACS. The percentage of cells positive for either CD4 or CD8 was multiplied by the number of total cells recovered from the tissue and the data displayed as the total number of either $CD4^+$ or $CD8^+$ cells (Figure 2). These data show that peripheral $CD4^+$ and $CD8^+$ T cell numbers were significantly, ($p < 0.001$), reduced over four days following *in vivo* treatment with 20 μg anti-CD3 antibody. The decrease in $CD4^+$ cells was statistically significant 72 ($p < 0.05$) and 96 h ($p < 0.001$) following anti-CD3 antibody treatment while the decrease in $CD8^+$ cells was statistically significant at 24, 72 h. ($p < 0.05$), and 96 h, ($p < 0.001$), post treatment. Curiously, there was no statistical differences in either T cell population 48 h following anti-CD3 antibody treatment.

Loss of peripheral T cells in superantigen models of T cell tolerance has been associated with activation of the responding T cells prior to their deletion (Kabelitz and Wesselborg.1992; Gonzalo *et al.*,1992; Webb *et al.*,1994; MacDonald *et al.*,1991; Renno *et al.*,1995). To determine if anti-CD3 antibody-induced T cell deletion was associated with T cell activation, increases in cell size and IL-2R expression of both $CD4^+$ and $CD8^+$ cells from 12-24 h after anti-CD3 antibody treatment were measured. There are many techniques to measure T cell activation, including increased forward light scatter (FSC) or cell size and

the use of antibodies to detect increased expression of cell surface activation markers such as IL-2R. Initial experiments measuring increases in FSC of $CD4^+$ splenocytes showed that the optimal difference between control-treated and anti-CD3 antibody-treated animals occurred 18 h following treatment. Therefore, increases in IL-2 R expression and FSC of $CD4^+$ and $CD8^+$ splenocytes from mice given anti-CD3 antibody 18 h before, at a dose which initiates T cell deletion (20 μg, high dose), and a dose which dose not lead to detectable T cell loss (0.1 μg, low dose), were measured. The high dose anti-CD3 antibody-treated mice had a significant increase in both cell size and IL-2R expression for $CD4^+$ and $CD8^+$ (not shown) splenocytes over hamster IgG-treated mice (Figure 3). These results suggest that the anti-CD3 antibody-induced T cell deletion was associated with prior cellular activation, as demonstrated by both increased forward light scatter and increased IL-2R expression of $CD4^+$ cells at the high dose but not the low dose of anti-CD3 antibody treatment. Other explanations for the deletion of splenic T cells which can not be ruled out by this type of experiment are T cell emigration and or sequestration. After treatment with anti-CD3 antibody, naive T cells express cell surface molecules which facilitate migration from circulation or lymphoid tissue into surrounding tissues in conjunction with the T cell cytokine induced endothelial cell expression of vascular adhesion molecules which allows the T cells to bind and traverse the endothelium (Bergese *et al.*,1994; Webb and Sprent.1993). H-2 alloreactive T cells home to the gut-associated lymphoid tissues with a significant portion of these cells eventually extruded into the gut lumen (Sprent.1976). Therefore, these mechanisms provide plausible explanations for the observed T cell depletion following anti-CD3 antibody treatment. These mechanisms would be less likely to be

important contributors to anti-CD3 antibody-induced T cell depletion if T cell death within the lymphoid organs could be demonstrated.

Anti-CD3 antibody-induced T cell deletion of $CD4^-$ $CD8^-$ T cells in the thymus was demonstrated to be primarily due to activation-induced cell death (Shi *et al.*,1991; McConkey *et al.*,1989; Groux *et al.*,1993). I confirmed that anti-CD3 antibody-treatment causes deletion of thymic T cells by demonstrating rapid reduction in the cellularity of the thymus between 12 and 48 h. Mice were treated with 20 μg of hamster IgG or anti-CD3 antibody 96, 72, 24 or 12 h before euthanasia. The thymocytes were recovered and labeled with antibodies to the cell surface markers CD4 and CD8, and the loss of $CD4^-$ and $CD8^-$ (double positive) cells was measured. This statistically significant, ($p < 0.001$), depletion was demonstrated to be rapid, resulting in a greater that 90% loss of double positive-thymocytes four days after treatment (Figure 4). The subsequent detection of fragmented oligonucleosomal-DNA 24 h after anti-CD3 antibody treatment from unsorted-thymic cells, suggested that the loss of thymocytes by apoptosis may be the same mechanism for the loss of peripheral T cells (Figure 5). If peripheral T cell apoptosis could be demonstrated it would make the hypothesis of T cell emigration or sequestration less likely an explanation for the observed T cell depletion. The demonstration of apoptosis of peripheral T cells *in vivo*following the administration of anti-CD3 antibody has not been documented. Therefore, it was critical to develop assays to demonstrate apoptosis, and provide evidence for the mechanism of anti-CD3 antibody-induced loss of peripheral T cells.

Figure 2. Kinetics of T cell depletion in the spleen and lymph nodes following anti-CD3 antibody treatment. BALB/c mice were given iv injections of 20 μg anti-CD3 antibody or control hamster IgG 96, 72, 48, 24 or 12 h before being euthanized. The tissue was removed and cells were counted on a Coulter counter and $2x10^6$ cells from each tissue were labeled with antibodies against the cell surface markers CD4 and CD8. The percent of $CD4^+$ or $CD8^+$ cells was calculated using a live gate analysis of cells multiplied by the total number of cells from the tissue. The mean (n=3) ± SEM for the number of recovered $CD4^+$ or $CD8^+$ positive lymphocytes is displayed. * $p < 0.05$, ** $p < 0.001$. These data are representative of two independent experiments.

Splenic T Cell Depletion Following *In Vivo* Treatment with Anti-CD3 Antibody

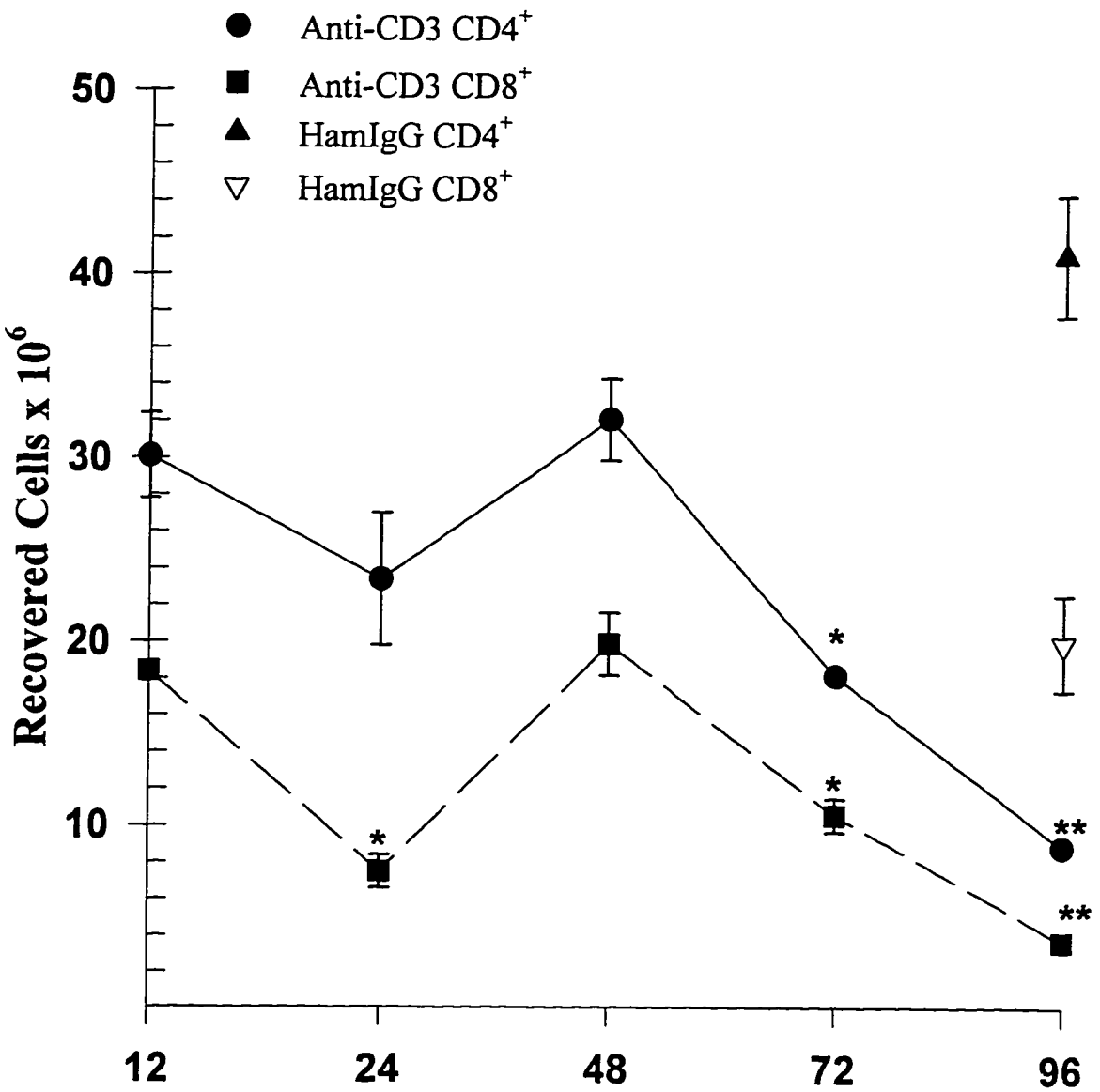


FIGURE 3. *In vivo* treatment with anti-CD3 antibody activates $CD4^-$ splenocytes. Mice were injected with 20 μg anti-CD3 or hamster IgG antibody (High Dose) or 0.1 μg anti-CD3 or hamster IgG antibody (Low Dose) and euthanized 18 h later. Cell suspensions were prepared from the spleen and the cells were surface labeled with antibodies against CD4 and IL-2R. The top portion of the figure depicts cell size data gated on $CD4^-$ cells. The bottom portion of the figure depicts IL-2R expression of $CD4^-$ cells. Data were collected on a Coulter EPICS Elite. Histograms are representative of one of two experiments conducted with three mice per treatment group.

Anti-CD3 Antibody Activates $CD4^+$ Splenocytes I*n Vivo*

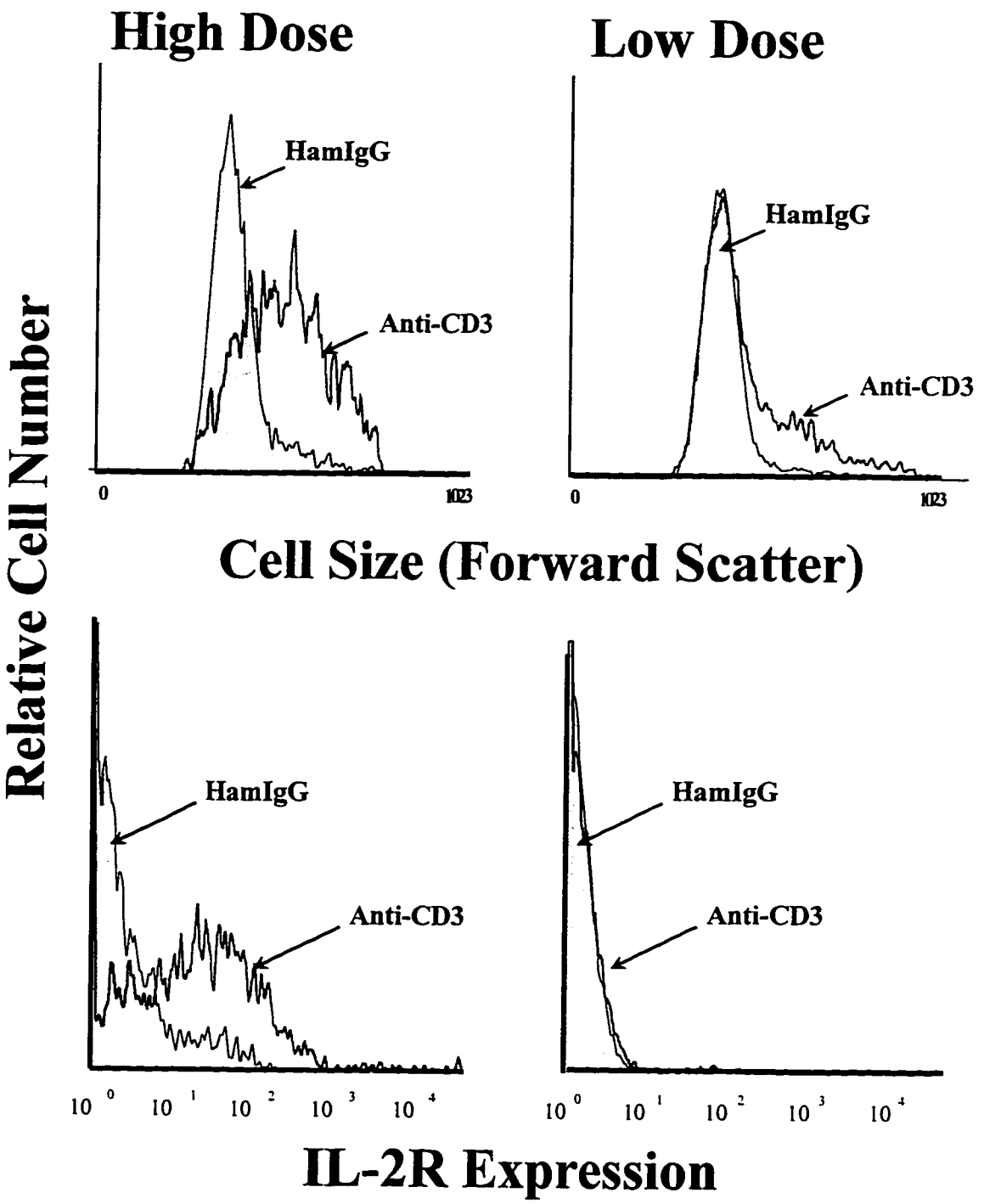


Figure 4. Kinetics of T cell depletion in the thymus following anti-CD3 antibody treatment. BALB/c mice were given iv injections of 20 μg anti-CD3 antibody or control hamster IgG 96, 72, 48, 24 or 12 h before euthanasia. The thymus was removed and cells were counted on a Coulter counter and $2x10^6$ cells from each sample were labeled with antibodies against the cell surface markers CD4 and CD8. The percent $CD4^+$ $CD8^-$ cells was calculated using a live gate analysis of the cells multiplied by the total number of cells from the tissue. The mean (n=3) ± SEM for the number of recovered $CD4^-$ $CD8^-$ positive thymocytes is displayed, * $p < 0.05$, ** $p < 0.001$. These data are representative of two independent experiments.

Thymic T Cell Depletion Following *In Vivo* Treatment With Anti-CD3 Antibody

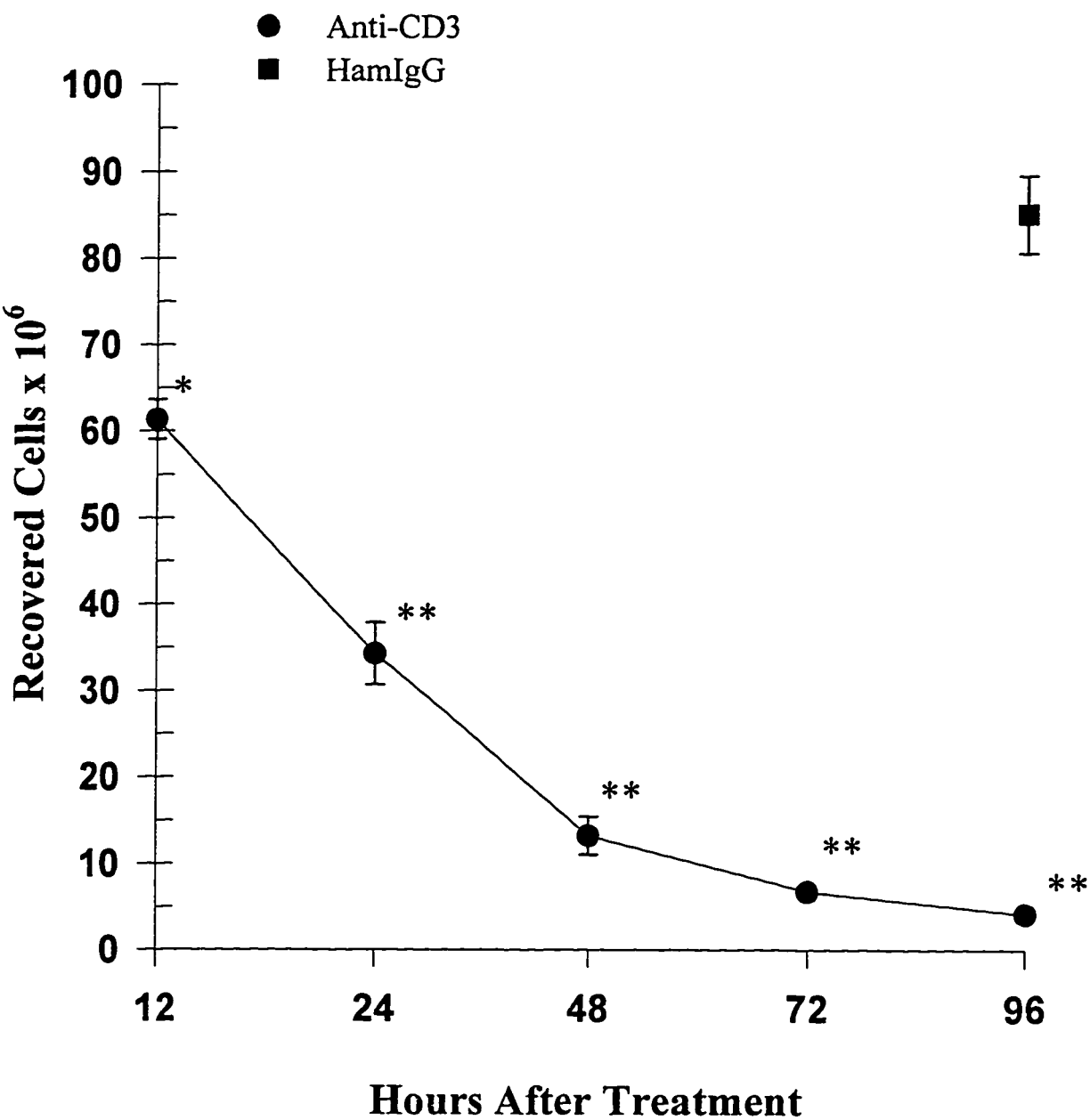
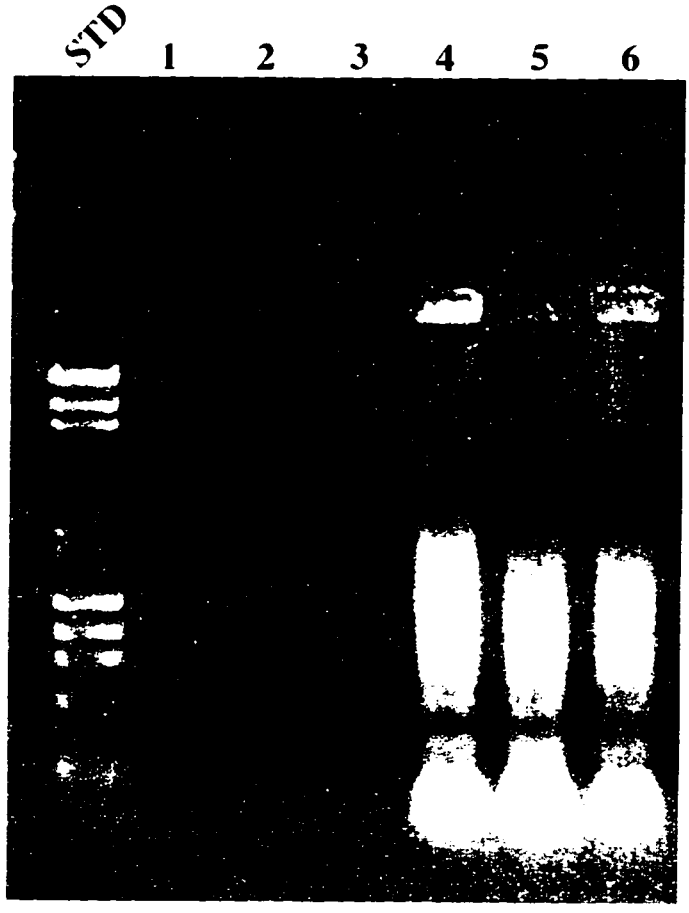


Figure 5. *In vivo* treatment with anti-CD3 antibody induces DNA fragmentation in thymocytes. Three mice per group were injected with either 20 μg 145-2C11 anti-CD3 antibody (lanes 4-6) or an equivalent amount of hamster IgG (lanes 1-3) and 48 h later the thymus was removed and cell suspensions were prepared. The cells were incubated for two h and approximately $1x10^6$ cells of each type were lysed in a hypotonic buffer. The lysate was centrifuged at 13,800 x g for 15 minutes. The supernatant, containing the small DNA fragments, was immediately removed and precipitated overnight. The following day the samples were treated with RNase and the DNA was electrophoresed on a 1X TAE gel. This experiment is representative of two independent experiments.

In Vivo Treatment with Anti-CD3 Antibody Induces Apoptosis of Thymocytes



In vivo treatment with anti-CD3 antibody leads to morphological changes characteristic of apoptosis in Thy 1$^+$ cells. The T cell receptor may be blocked and/or modulated from the surface of T cells following *in vivo* treatment with anti-CD3 antibody and thus prevent detection of T cells undergoing apoptosis with antibodies directed at the TCR or its associated CD4 or CD8 markers (Hirsch *et al.*, 1989). Therefore, a surface marker (Thy1.2) not associated with the TCR was chosen to identify T cells following administration of anti-CD3 antibody. The depletion of peripheral T cells and subsequent T cell tolerance to transplantation antigens following *in vivo* treatment with anti-CD3 antibody is the only evidence provided, thus far, that peripheral T cells undergo apoptosis (Hirsch *et al.*, 1988; Hirsch *et al.*, 1990; Hirsch *et al.*, 1991; Bluestone *et al.*, 1993; Janssen *et al.*, 1992). Cells undergoing apoptosis *in vivo* are rapidly removed by professional and nonprofessional phagocytes during the early stages of apoptosis (Cohen *et al.*, 1992; Gavrieli *et al.*, 1992; Surh and Sprent.1994; Squier *et al.*, 1995). This fact, plus the limited assays for detecting apoptosis, may explain the lack of direct *in vivo* evidence for anti-CD3 antibody-induced apoptosis. In an attempt to explain the previous observations of T cell depletion following administration of mitogenic anti-CD3 antibody, adaptations of current assays for the detection of apoptosis of Thy1$^+$ cells *in vivo* were undertaken.

Cells *in vitro*, undergoing apoptosis, exhibit morphological changes such as condensation of nuclear chromatin, structural changes in the cell membrane and fragmentation of DNA into oligonucleosomal fragments (Kabelitz *et al.*, 1993; Gerschenson and Rotello.1992; Cohen *et al.*, 1992). Initial studies were unable to detect genomic DNA fragmentation in splenic T cells after treatment with 20-30 μg of anti-CD3 antibody.

Therefore, the addition of a short term *ex vivo* culturing step was included prior to assaying splenocytes from anti-CD3 antibody-treated mice for apoptosis. This step was added for several reasons; 1) apoptotic cells are rapidly phagocytosed *in vivo* and can be detected inside macrophages (Surh and Sprent.1994); 2) the process is noninflammatory suggesting that it occurs prior to release of cellular contents (Cohen *et al.*, 1992; Squier *et al.*, 1995); 3) DNA fragmentation in anti-CD3 antibody treated T cell hybridomas is blocked by inhibitors of transcription and translation (Ucker *et al.*, 1989) suggesting that time is required for the expression of detectable manifestations of apoptosis mediated by new apoptotic products; 4) detection of apoptosis following anti-CD3 antibody stimulation of activated T cells *in vitro* required a short term culturing step (Jenkins *et al.*, 1990; Groux *et al.*, 1993); and 5) detection of apoptosis in dexamethasone treated thymocytes via the TUNEL assay was improved with a short term culturing step (Dr. Leslie King, NIH, personal communication). Preliminary experiments to detect apoptosis of splenocytes from mice treated two days prior with anti-CD3 antibody and then incubated for 2-24 h were completed. These experiments revealed that incubation of the cells for 2-4 h *ex vivo* led to the optimal appearance of fragmented DNA in lymph node cells in anti-CD3 antibody-treated but not control-treated mice (data not shown).

The two-hour incubation was subsequently used to aid detection of cells exhibiting the morphological changes associated with apoptosis. This incubation may provide time for the manifestation of easily detectable apoptotic characteristics such as nuclear condensation and DNA fragmentation by disrupting the normal architecture of cell to cell interactions that facilitate the removal of apoptotic cells (Webb and Sprent.1993). Following *in vivo*

treatment with anti-CD3 antibody, Thy 1^+ splenocytes and lymph node cells were examined for morphological changes associated with apoptosis by two techniques. The first technique detected fragmentation of DNA into oligonucleosomal fragments, a hallmark of apoptosis, and the second technique detects nuclear condensation of apoptotic cells by fluorescent microscopy (Cohen *et al.*, 1992; Mishell *et al.*, 1980; Coligan 1991).

Splenocytes or lymph node cells were obtained from mice two days after injection with 20 μg hamster IgG or anti-CD3 antibody, incubated for two h, labeled with an anti-Thy1 antibody, and sorted into Thy1 positive and negative populations. The cellular nucleic acid was stained with the vital dye ethidium bromide and the supravital dye acridine orange. Viable cells that possess a membrane potential will exclude ethidium bromide but will take up acridine orange. The DNA will fluoresce green and the RNA may appear red when stimulated with UV light; conversely the DNA of nonviable cells will stain with ethidium bromide and appears orange (Coligan 1991). Treatment with anti-CD3 antibody induced characteristics associated with apoptosis in Thy1^+ splenocytes (Figure 6). These data show viable (Figure 6. a, b) and nonviable (Figure 6. c, d) cell populations based on their ability to exclude ethidium bromide. These T cells can then be classified as normal (Figure 6. a, c), based on large nuclear morphology with diffuse chromatin, or apoptotic (Figure 6. b, d), based on a condensed nuclear morphology and compacted chromatin. Thus, it was determined that lymph node cells from mice treated with anti-CD3 antibody had eight times as many Thy1^+ apoptotic cells as mice given isotype control antibody (Table 3). The increased apoptosis of Thy1^+ cells following anti-CD3 antibody treatment was statistically significant ($p < 0.001$).

The second technique was used in a similar experiment to determine if chromatin degradation and cleavage of DNA by endogenous endonuclease into 180-200 bp fragments, which is associated with activation-induced apoptosis, occurred in Thy1$^+$ cells. Thy1$^+$ lymph node cells were prepared and incubated as described above and cell lysates were examined for low molecular weight DNA which appears as a ladder in 200 bp multiples after electrophoresis through an agarose gel. Following *in vivo* treatment with anti-CD3 antibody, but not control hamster IgG antibody, DNA from Thy1$^+$ but not Thy1$^-$ lymph node cells underwent cleavage associated with apoptosis (Figure 7). The cleavage was only apparent in the Thy1$^+$ cells from anti-CD3 antibody-treated mice; which demonstrates the specificity of induction of apoptosis in the Thy 1$^+$ population. Analysis of lymph node cells from untreated mice showed that greater than 96% of Thy 1$^+$ cells were also CD3$^+$. This assay is extremely useful for determining if a cell population has undergone activation induced cell death since DNA fragmentation appears early in the process and occurs in most activation-induced cell death models (Mogil *et al.*, 1994). A major disadvantage is that the assay is not quantitative and requires a large number of cells to reliably detect fragmentation ($1x10^6$ cells) suggesting that the sensitivity of the assay may be limiting.

FIGURE 6. *In vivo* administration of anti-CD3 antibody leads to morphological changes characteristic of apoptosis in Thy 1.2^+ cells. Mice were injected with 20 μg of 145-2C11 anti-CD3 antibody and were euthanized 48 h later. The spleens were removed and cell suspensions prepared. The cells were incubated in media for two h, labeled with biotinylated anti-Thy1 antibody followed by SA-PE and sorted into positive and negative populations. The sorted cells were stained with a mixture of acridine orange and ethidium bromide to differentiate viable and nonviable cells and examined by fluorescent microscopy for apoptotic morphology. Figures a-d are representative cells from the Thy1.2^+ population: (a) viable nonapoptotic, (b) viable apoptotic, (c) nonviable nonapoptotic, (d) nonviable apoptotic. Numerical data from one of three experiments are displayed in Table 3.

In Vivo Administration of Anti-CD3 Antibody Leads to Morphological Changes Characteristic of Apoptosis in Thy1$^+$ Cells

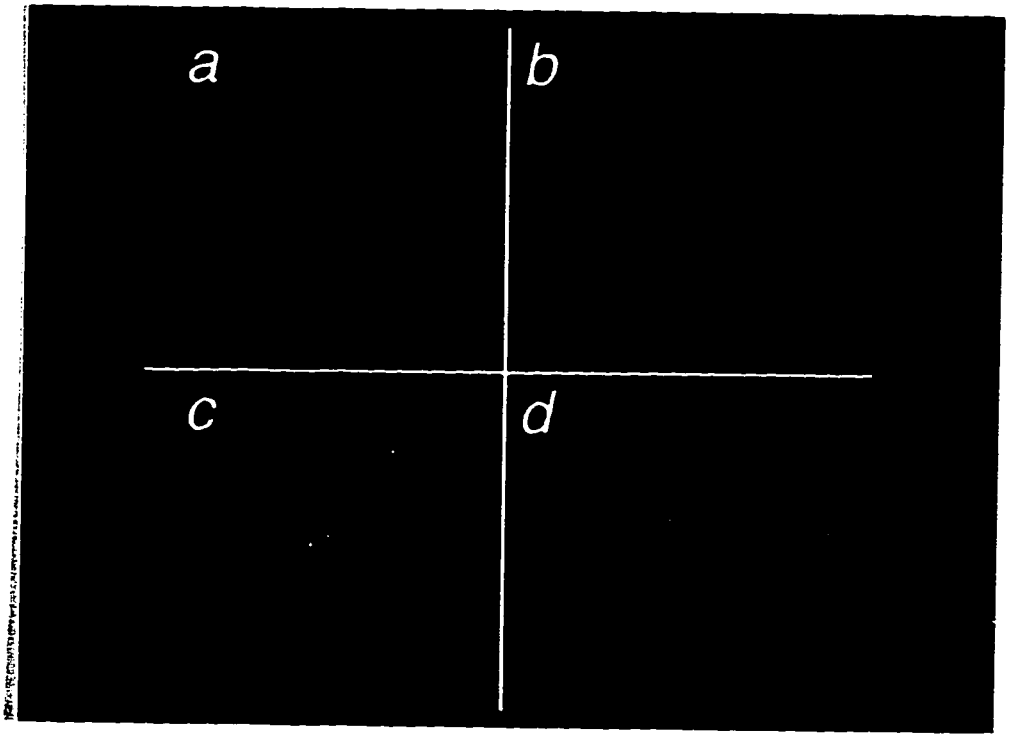


Table 3. Quantitation of Apoptotic Lymph Node T cells 48 Hours Following *In Vivo* Administration of Anti-CD3 Antibody.

ANTIBODY TREATMENT[a]	SORT[b]	MEAN % APOPTOTIC[c]
hamster IgG	**THY 1.2$^-$**	**0.8 ± 0.05**
	THY 1.2$^+$	**1.1 ± 0.1**
anti-CD3	**THY 1.2$^-$**	**1.3 ± 0.7**
	THY 1.2$^+$	**9.1 ± 0.9****

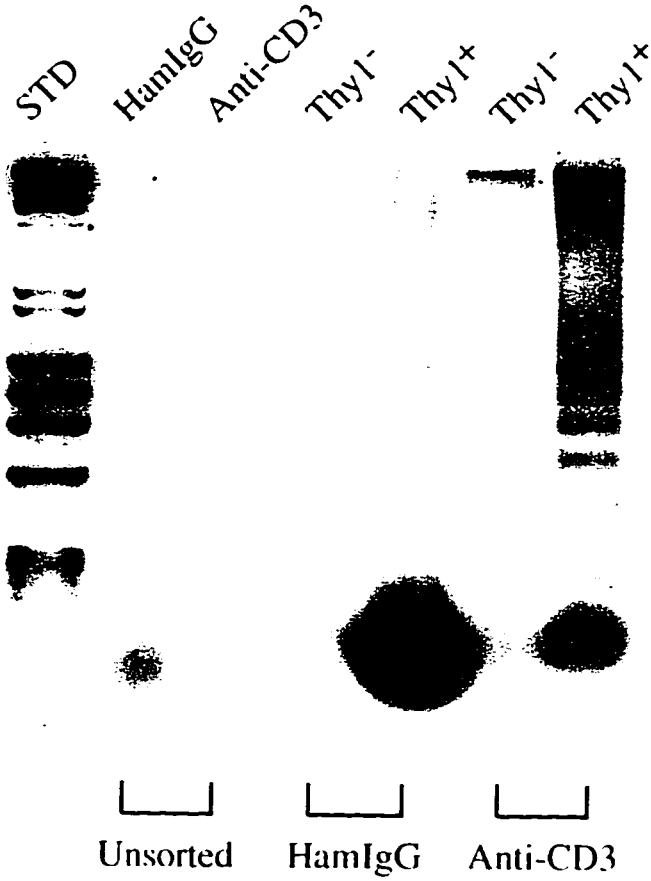
[a] Treatment as described in the Materials and Methods section.

[b] Pooled lymph node cells were stained with biotinylated anti-Thy1.2 followed by streptavidin-PE and were sorted with a Coulter EPICS Elite. Populations were determined to be greater than 98% pure upon reanalysis.

[c] Cells were quantitated by staining with acridine orange and ethidium bromide followed by examination for apoptotic morphology by fluorescent microscopy. Both viable and nonviable apoptotic cells were included in the calculation. The mean and standard error were determined for a triplicate sample of the pooled, sorted cells consisting of five animals per group, ** $p < 0.001$ (compared with hamster IgG treated Thy1.2$^-$ cells). A minimum of 1,500 cells were counted for each sorted group. These data are representative of one of three experiments.

FIGURE 7. Anti-CD3 antibody treatment *in vivo* induces DNA fragmentation in peripheral Thy1$^+$ cells. Five mice per group were injected with either 20 μg 145-2C11 anti-CD3 antibody or an equivalent amount of hamster IgG and 48 h later brachial and axillary lymph node pairs were removed and cell suspensions were prepared. The cells were incubated for two h and labeled with anti-Thy1 antibody, then sorted into positive and negative populations. Approximately $1x10^6$ cells of each type were lysed in a hypotonic buffer. The lysate was centrifuged at 13,800 x *g* for 15 minutes. The supernatant containing the small DNA fragments was immediately removed and precipitated overnight. The following day the DNA was electrophoresed on a 1X TAE gel. These results are representative of one of three experiments.

Anti-CD3 Antibody Treatment Induces DNA Fragmentation in Peripheral Thyl$^+$ Cells



Anti-CD3 antibody treatment in vivo induces a detectable decrease in size of a subpopulation of Thy1$^-$ cells and a corresponding increase in DNA fragmentation. Another characteristic of cells undergoing apoptosis is a reduction in cell size detectable as a decrease in FSC by FACS analysis (Chrest *et al.*, 1993; Zamai *et al.*, 1993). An examination of the FSC *vs.* SSC profile of splenocytes from mice treated *in vivo* with 20 μg of anti-CD3 antibody revealed a subpopulation with reduced FSC (Figure 8.1). If this population contained apoptotic cells then they should have fragmented DNA. Free 3' ends of the DNA fragments can be detected by a nick translation assay using FITC-labeled dUTP (Gavrieli *et al.*, 1992; Darzynkiewicz *et al.*, 1992; Hotz *et al.*, 1994; Gorczyca *et al.*, 1993). The incorporation of dUTP in anti-CD3 antibody-treated lymphocyte subpopulations is shown in Figure 8.2-8.4. These data demonstrate that incorporation of UTP occurs in a cell population with reduced size (Figure 8.3), and that this incorporation is specific since viable lymphocytes incorporated very little UTP (Figure 8.4). As a control for nonspecific UTP incorporation due to random DNA breaks, the UTP profile of splenocytes from anti-CD3 antibody-treated mice was compared to splenocytes that were heat killed. Treatment of cells for two h at 42^{o}C induces death by necrosis rather that apoptosis . Death by necrosis is associated with random breaks in the DNA (Cohen *et al.*, 1992; Dive *et al.*, 1992). Overlapping the UTP histograms from the anti-CD3 antibody-treated and heat killed splenocytes shows that death by necrosis induces random DNA breaks which produce a broad FITC^{dull} population of less intensity as compared to the brightly staining well defined FITC^{high} apoptotic population (Figure 8.5).

To demonstrate that the cell population with reduced FSC were T cells, experiments were conducted combining cell-surface labeling with anti-Thy 1 antibody, and detection of DNA fragmentation (apoptosis) by dUTP incorporation. Mice were injected with 20 μg of hamster IgG or anti-CD3 antibody and two days later the spleens were removed and incubated *ex vivo* for two h. The cells were labeled with anti-Thy1 antibody and the free 3' DNA ends were labeled with UTP as described above. The resulting fluorescent profiles of both markers were analyzed. These data show that anti-CD3 antibody-treated mice have an approximate three-fold increase in apoptotic Thy1$^-$ cells compared to control treated mice (Figure 9) . The histograms show a population of Thy 1^{int} FITC$^-$ cells which represent the apoptotic cells. Significant apoptosis of Thy1$^-$ cells by this method could be detected from 24-72 h following treatment with anti-CD3 antibody. The peak time for detecting the greatest difference between control-treated and anti-CD3 antibody-treated splenocytes was 48 h. This was determined by multiplying the number of Thy1$^-$ cells by the percent of Thy1$^-$ cells also positive for UTP. The reduction in Thy1 labeling is most likely due to a general loss of cell surface markers during apoptosis associated with known changes in the lipid bilayer (Cohen *et al.*, 1992; Squier *et al.*, 1995). Splenocytes were examined at earlier time points for the loss of other cell-surface markers. CD4$^-$ splenocytes had a rapid, significant reduction in TCR expression. Approximately 40% of these T cells from anti-CD3 treated mice have blocked/modulated or down regulated TCR$\alpha\beta$ expression (Figure 9). The loss of cell-surface CD3 complexes was similar to the loss of the TCR.

FIGURE 8. Peripheral FITC-UTP positive cells from anti-CD3 antibody-treated mice have reduced forward light scatter and do not represent a necrotic population. Histograms 1-4 depict splenocytes following *in vivo* treatment with 20 μg of anti-CD3 antibody or hamster IgG as described in the legend to Figure 6. The cells were fixed overnight in formaldehyde and permeated with methanol the following day. Free 3' DNA ends were labeled with FITC conjugated dUTP by DNA polymerase one. Histogram 1 shows FSC vs. SSC with gates B representing viable lymphocytes, C representing lymphocytes with reduced FSC, and gate A which includes gates B, C. Histogram 2 shows the FITC-UTP profile of gate A. Histogram 3 represents the FITC-UTP profile of smaller "apoptotic" cells and histogram 4 represents viable cells. Histogram 5 compares the FITC-UTP profiles of splenocytes from gate A to untreated splenocytes which were heat killed by incubating at 42^{o}C for two h. These data are representative of two experiments.

Anti-CD3 Antibody Treatment Increases DNA Fragmentation In Splenocytes With Reduced Forward Light Scatter

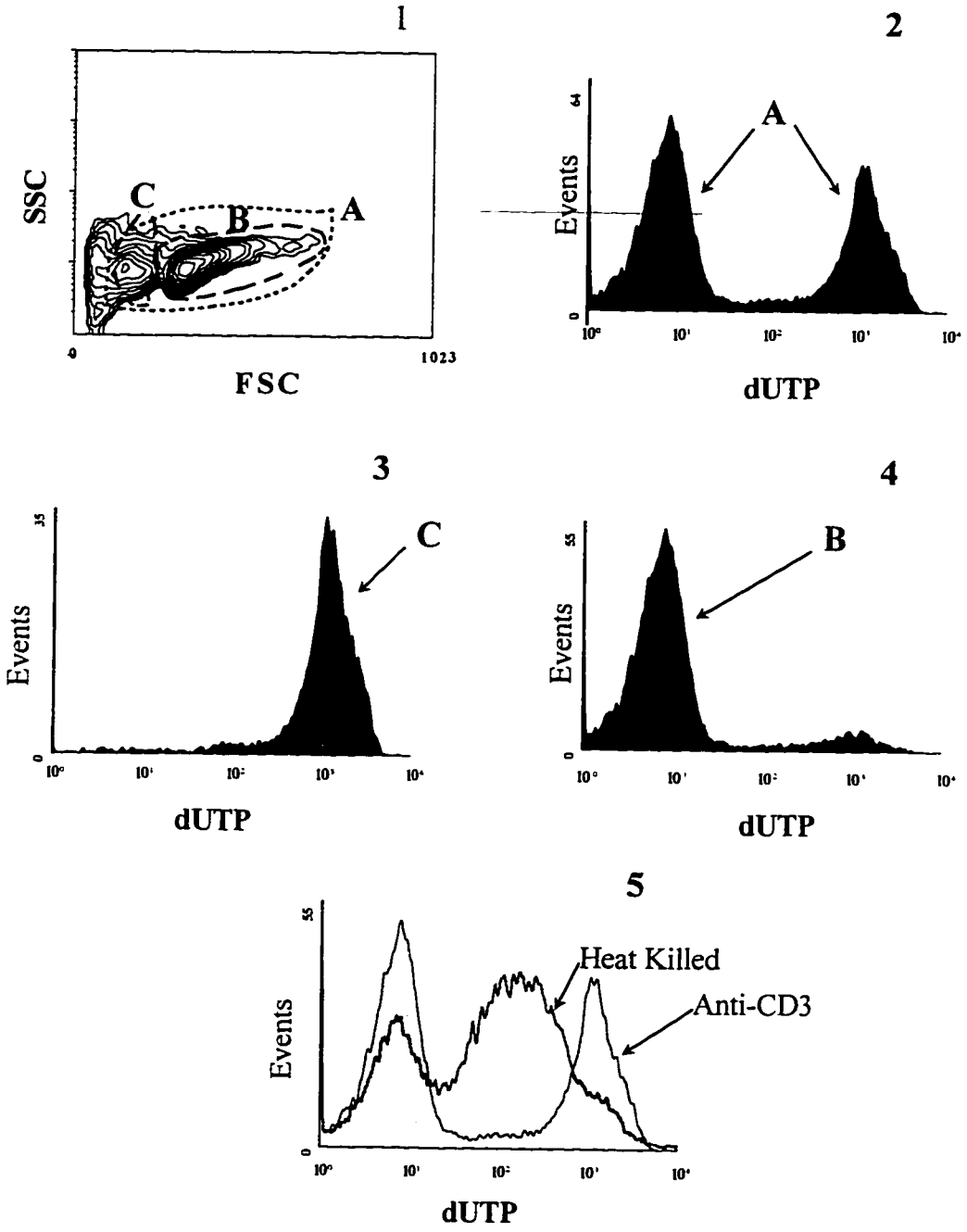
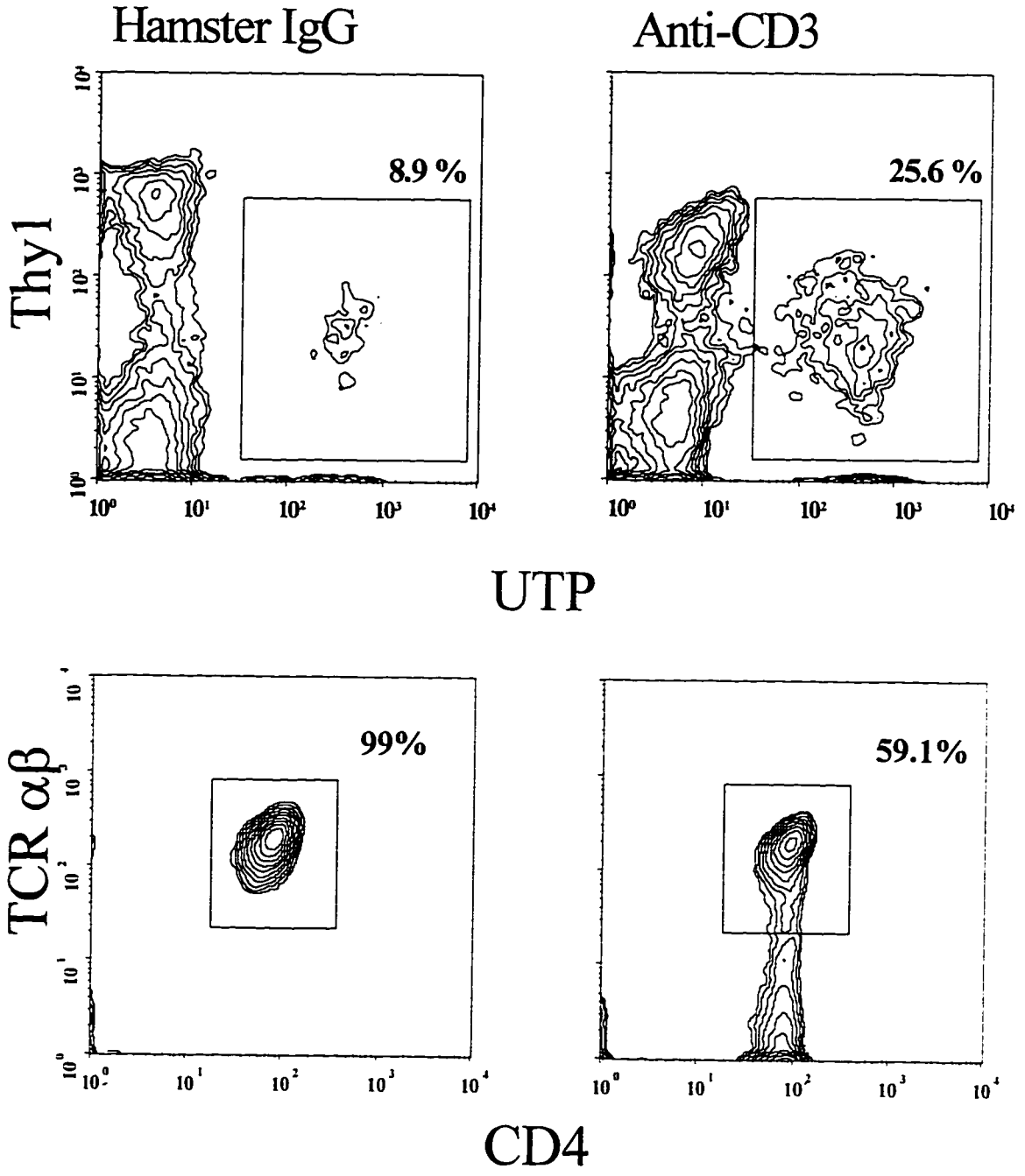


FIGURE 9. Anti-CD3 antibody treatment *in vivo* induces changes in cell surface markers and a detectable increase in apoptosis of Thy1$^-$ cells. Mice were treated as described in the legend to Figure 6. The spleens were removed and cell suspensions were prepared. The top portion of the figure shows histograms of cells that were incubated for two h and then surface labeled with biotinylated anti-Thy1 antibody followed by streptavidin CY-Chrome. After surface labeling the cells were fixed overnight in 2% formaldehyde. The following day the cells were permeated with methanol, washed, and added to a nick translation assay. The incorporation of FITC conjugated dUTP was analyzed using a Coulter EPICS Elite. Representative histograms for a control-treated and anti-CD3 antibody-treated spleen cells are displayed. Data collected from a representative experiment are presented in Table 4. The bottom portion of the figure represents data collected from a similar experiment. Splenocytes from this experiment were harvested 1 h following treatment, and were not incubated, but instead immediately labeled with anti-CD4-FITC and anti-TCR$\alpha\beta$-PE. CD4$^-$ splenocytes were sorted with a Coulter EPICS Elite and analyzed for TCR staining. Numbers represent the percent of cells which are positive for both markers.

Changes in Splenic T cell Surface Markers Following *In Vivo* Treatment With Anti-CD3 Antibody



Part II. Cell surface phenotype of cells undergoing anti-CD3 antibody induced apoptosis.

In vivo administration of anti-CD3 antibody leads to the accumulation of abnormal cells, similar to cells present in lpr mice. To demonstrate that the Thy1 staining of apoptotic cells was specific and not an artifact of surface changes noted to occur when cells undergo apoptosis, a three color FACS analysis was performed. Lymphocytes from anti-CD3 antibody-treated mice were surface labeled with antibodies to the T cell marker Thy1 and the B cell marker B220, and fragmented DNA was internally labeled with dUTP. This experiment revealed three populations of apoptotic cells which incorporated UTP and stained for surface markers in the following combinations: $Thy1^-$ $B220^-$, $B220^-$ $Thy1^-$; or $Thy1^-$ $B220^-$ (Figure 10). Isotype control staining was negative, arguing against activation-induced nonspecific binding by anti-CD3 antibody-treated cells. $B220^-$ $Thy1^-$ UTP^- cells were gated out of the analysis and the percent of cells which were $Thy1^-$ $B220^-$ UTP^- were recalculated and expressed as an increase over control antibody-treated animals. A ten-fold increase in apoptotic cells in the spleens and a seven fold increase in the lymph nodes of anti-CD3 antibody-treated mice over control-treated mice was found (Table 4). These increases were statistically significant ($p < 0.001$). Thy1 is generally expressed only by T cells but *in vitro* experiments, using LPS-stimulated B cells, show that IL-4 induces B cells to express Thy1 mRNA and surface protein (Snapper.1990; Snapper *et al.*,1988). In addition, it was demonstrated that *in vivo* treatment with goat anti-mouse IgD antibody leads to the production of IL-4, and subsequent class switching of B cells to IgE producing cells, of

which a large portion also express Thy1. These data suggest an important role for IL-4 in the induction of Thy1 in B cells (Snapper *et al.*, 1990). Since *in vivo* treatment with anti-CD3 antibody also induces a significant amount of IL-4 expression (Flamand *et al.*, 1990; Scott *et al.*, 1990), which may initiate Thy1 expression on B cells, a four color FACS analysis was performed to determine the cell lineage of the $Thy1^{+}$ $B220^{+}$ apoptotic lymphocytes. Splenic cells from anti-CD3 antibody-treated and control antibody-treated mice were surface labeled with antibodies to Thy1, B220 and IgM and internally labeled with UTP. The gates were set on apoptotic UTP^{+} IgM^{+} cells and the B220 vs. Thy1 staining profile was examined (Figure 11). These data demonstrate that although there are $B220^{+}$ UTP^{+} IgM^{+} cells in control antibody-treated mice, the majority of UTP^{+} IgM^{+} cells are B220 $Thy1^{+}$ in anti-CD3 antibody-treated mice. A second experiment using four color FACS analysis confirmed these results. IgM^{+}, UTP^{+} splenocytes were examined for Thy1 *vs.* B220 staining. The results of this experiment show splenocytes treated with anti-CD3 antibody are $Thy1^{+}$ $B220^{+}$ (Figure 12).

To exclude the possibility that these apoptotic cells were B cells expressing Thy1, a multi-parameter three color experiment was repeated in IgM knockout mice. These mice have a disruption of the membrane spanning exon of Cμ and homozygotes have no mature surface $B220^{+}$ IgM^{+} or IgD^{+} B cells in the blood, spleen, bone marrow, or peritoneal cavity and lack detectable serum IgM antibody (Kitamura *et al.*, 1991). Anti-CD3 antibody treatment of IgM knockout mice induces statistically significant, ($p < 0.001$), changes in preapoptotic and apoptotic cells. These changes include a three-fold increase in the percent of lymphocytes which are $Thy1^{+}$ $B220^{+}$ UTP^{+} and $Thy1^{+}$ $B220^{+}$ UTP^{+}, a four-fold increase

in Thy1$^-$ B220$^-$ UTP$^-$ lymphocytes, and a nine-fold increase in Thy1$^-$ B220$^-$ UTP$^-$ lymphocytes (Table 5). Thy1$^-$ B220$^-$ cells similar to this phenotype which are also negative for the T cell markers CD4 and CD8 have been reported to accumulate in mice with genetic defects in *fas* and *fasL* known as *lpr* and *gld* mutations, respectively (Wu *et al.*, 1994). In a separate analysis we confirmed that B220$^+$ Thy1$^-$ from anti-CD3 antibody-treated mice were CD4$^-$, CD8$^-$ (Figure 13). The results of these experiments suggest that the B220$^-$ Thy1$^-$ UTP$^-$ cells are in fact apoptotic T cells with a phenotype similar to the aberrant lymphocytes that accumulate in *lpr* and *gld* mice. It also suggests that the B220$^-$ UTP$^-$ cells are T cells that do not express or have down-regulated expression of Thy1 as previously observed in wild type mice (Figure 9).

FIGURE 10. *In vivo* treatment with anti-CD3 antibody leads to the development of apoptotic cells which are $B220^{-}$ $Thy1^{-}$. Mice were injected with 20 μg anti-CD3 or hamster IgG antibody then euthanized 48 h later. Cell suspensions were prepared from the spleen or lymph nodes (not shown) and the cells were incubated for two h. Following culture the cells were surface labeled with antibodies against B220 and Thy1 and prepared for nick translation as described in the legend to Figure 9. The following day the fragmented DNA was labeled with UTP and the three-color FACS analysis completed on a Coulter EPICS Elite. The histograms are representative of one of two experiments using three/five mice per group respectively. Histogram scale is logarithmic.

In vivo Treatment With anti-CD3 Antibody Leads to the Development of $B220^{+}$ $Thy1^{+}$ Apoptotic Cells

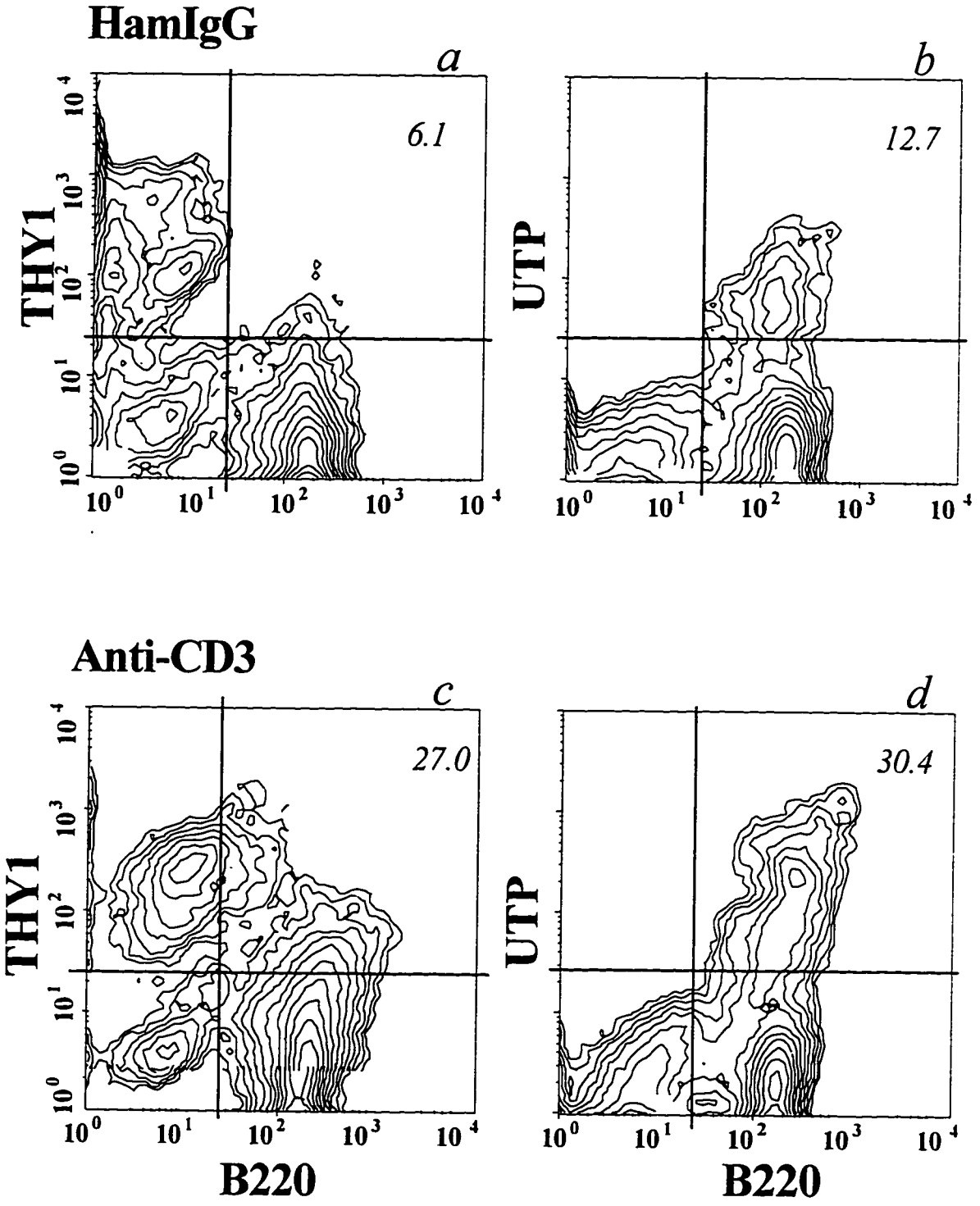


TABLE 4. Increase in Apoptosis of Peripheral Thy1$^-$ Cells 48 Hours Following *In Vivo* Administration of Anti-CD3 Antibody.

Tissue	Treatment	Mean % Apoptotic[a]	Fold Increase[b]
Spleen[c]	hamster IgG	1.5 ± 0.05	
	anti-CD3	15.2 ± 0.7**	10.1
Nodes[d]	hamster IgG	1.7	
	anti-CD3	12.4**	7.3

[a] The mean was based on four animals per treatment group and is expressed as the percent of cells staining positive for both Thy1 and FITC-UTP excluding B220$^-$ Thy1$^-$ cells ± SEM,** $p < 0.001$ (compared to hamster IgG treated tissues).

[b] The fold increase represents the percent increase in detectable apoptosis over background apoptosis seen in control-treated mice given hamster IgG, calculated by dividing the mean anti-CD3 antibody-treated percent by the mean control antibody-treated percent.

[c,d] Tissue was collected and prepared as described in the materials and methods. Brachial and axillary lymph nodes for each treatment group were pooled. These data are representative of three experiments for splenic gene expression and two experiments for lymph node gene expression.

FIGURE 11. *In vivo* treatment with anti-CD3 antibody leads to the development of apoptotic cells which are IgM^- $B220^-$ $Thy1^+$. Mice were injected with 20 μg anti-CD3 antibody and euthanized 48 h later. Cell suspensions were prepared from the spleen and the cells were incubated for two h. Following incubation the cells were surface labeled with antibodies against IgM, B220 and Thy1 and prepared for nick translation as described in the legend to Figure 9. The following day the fragmented DNA was labeled with UTP and the four-color FACS analysis completed on a Coulter EPICS Elite. The histograms depicted are representative of one of two experiments with three mice per treatment group. Gates were established on UTP^+ IgM^- cells and the resulting B220 *vs.* Thy1 fluorescent profiles were analyzed. The histogram scale is logarithmic.

Anti-CD3 Antibody Treatment Induces B220 Expression In $Thy1^+$, IgM^-, UTP^+ Cells

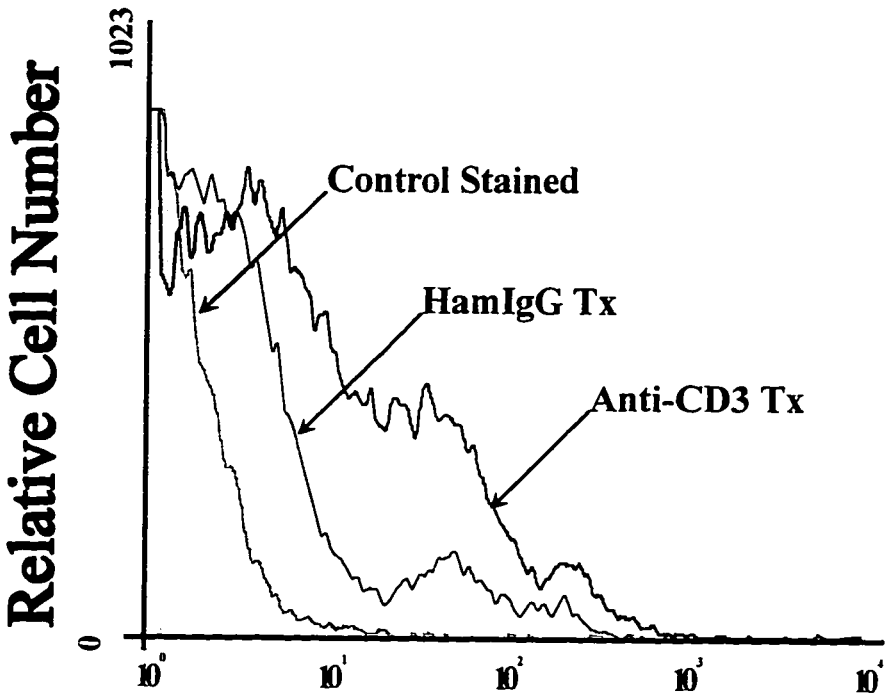


FIGURE 12. *In vivo* treatment with anti-CD3 antibody leads to the development of apoptotic cells which are IgM^- $B220^+$ $Thy1^+$. See the legend to Figure 11. Gates were established on UTP^+ IgM^- cells and the resulting B220 *vs.* Thy1 fluorescent profiles were analyzed. The histogram scales are logarithmic. These data are representative of one of two experiments.

In Vivo Treatment with Anti-CD3 Antibody Induces Apoptosis In Thy1^{+}, B220^{+}, IgM^{-} Cells

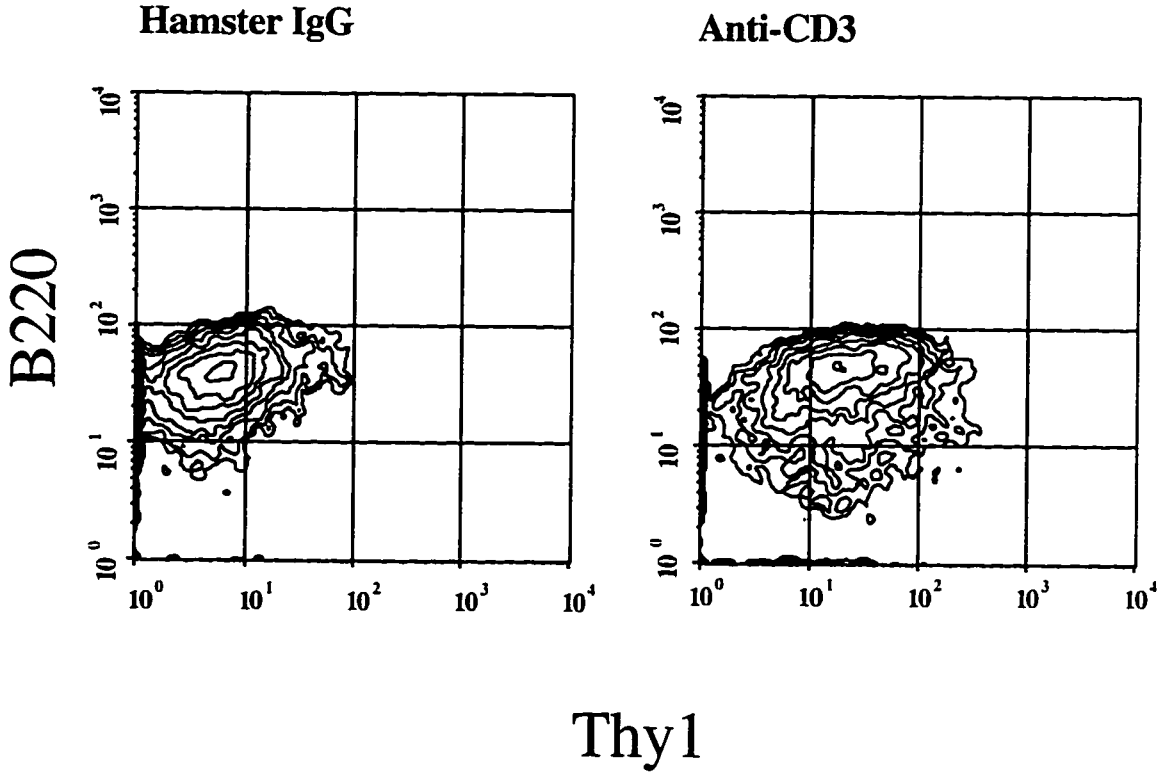


Table 5. Cell Surface Phenotype of Splenic Lymphocytes in IgM Knockout Mice.

	Mean ± SEM % Lymphocyte Staining[a]					
Group[b] Treatment	%Thy1$^-$ B220$^+$ UTP$^-$	%Thy1$^+$ B220$^-$ UTP$^-$	%Thy1$^+$ B220$^+$ UTP$^-$	%Thy1$^-$ B220$^+$ UTP$^+$	%Thy1$^+$ B220$^-$ UTP$^+$	%Thy1$^-$ B220$^-$ UTP$^+$
B6 hamIgG	51.9 ± 3.6	23.2 ± 1.9	1.3 ± 0.5	7.3 ± 1.9	1.3 ± 0.2	1.4 ± 0.1
B6 anti-CD3	41.9 ± 2.7	22.4 ± 2.8	5.8 ± 0.4**	4.4 ± 0.7	5.4 ± 0.3**	14.9 ± 1.2**
B6 IgM^{KO} hamIgG	0.7 ± 0.1	47.6 ± 0.7	1.2 ± 0.4	5.5 ± 1.0	2.1 ± 0.2	2.0 ± 0.6
B6 IgM^{KO} anti-CD3	0.6 ± 0.1	40.6 ± 1.4	5.0 ± 0.8**	20.3 ±2.0**	7.4 ± 0.5**	17.8 ± 2.4**

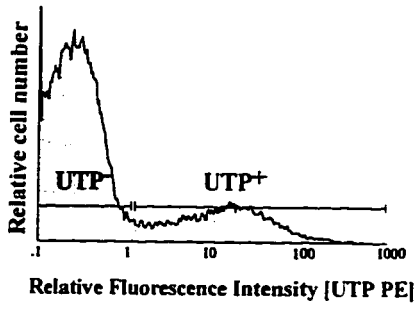
[a] Splenic cell preparations were stained with anti- B220-FITC and anti-Thy1-PE then free 3' DNA ends were labeled with biotinylated dUTP followed by SA-APC. Gates were established to exclude monocytes, RBCs, cell doublets and debris and to include lymphocytes and lymphocytes with reduced FSC. Mean and SEM for B-6 mice, three per group, and B-6 IgM^{KO} mice, five per group, are displayed for each group.** $p < 0.001$ (compared to hamster IgG treated mice of the same genetic background).

[b] Mice were given iv injections of 20 μg hamster IgG or anti-CD3 antibody and 48 h later the mice were euthanized.

Figure 13. *In vivo* treatment with anti-CD3 antibody leads to the development of apoptotic cells which are $B220^+$ $Thy1^-$ $CD4^-/CD8^-$. Mice were injected with 20 μg anti-CD3 antibody and euthanized 48 h later. Cell suspensions were prepared from the spleen and incubated for two h. Following incubation the cells were surface labeled with antibodies against B220, Thy1, CD4 or CD8 and prepared for nick translation as described in the legend to Figure 9. The following day the fragmented DNA was labeled with UTP and the four-color FACS analysis completed on a Coulter EPICS Elite. The histograms depicted are representative of one of two experiments with three mice per treatment group. Gates were established on UTP^+ or UTP^- cells and the resulting B220 *vs.* Thy1/CD4/CD8 fluorescent profiles analyzed. The histogram scales are logarithmic.

Apoptotic Splenocytes are Thy1^{+}, B220^{+}, CD4^{-}, CD8^{-}.

A



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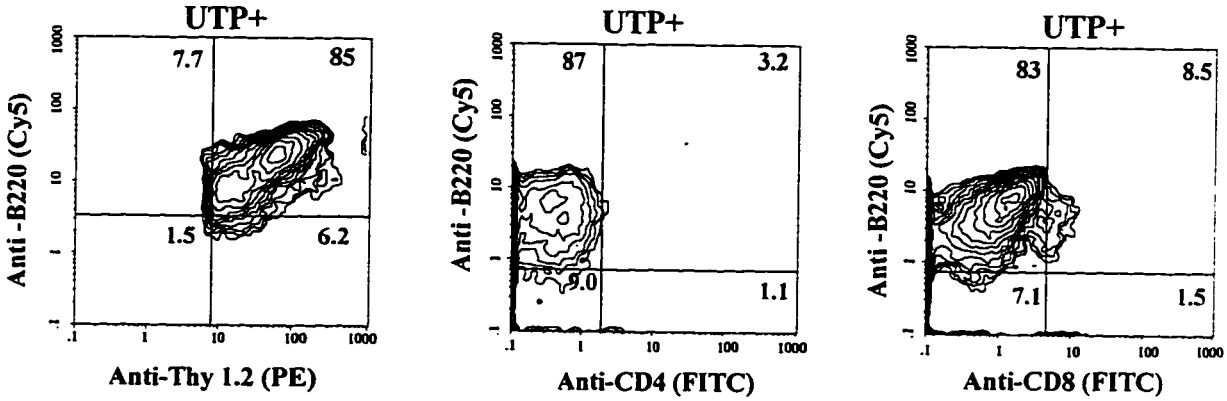

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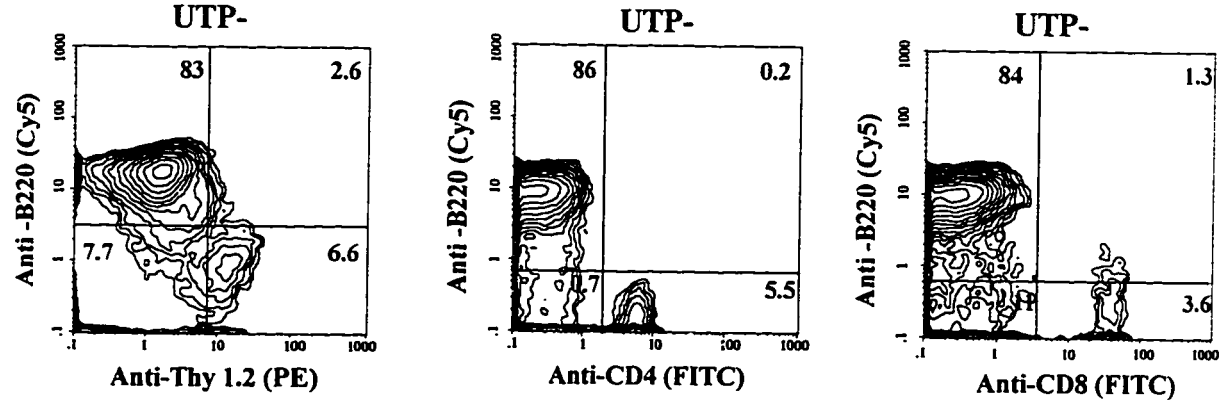


Administration of anti-CD3 antibody in vivo induces rapid fas and fasL but not bcl-2 gene expression. The expression of *fas* and *fasL* has been associated with increased susceptibility of T cells to apoptosis while the expression of *bcl-2* has been associated with resistance to apoptosis (Crispe.1994; Itoh *et al.*, 1993). To determine if anti-CD3 antibody-treatment increased expression of *fas*, *fasL* or *bcl-2*, mice were injected with 20 μg of anti-CD3 antibody or hamster IgG antibody then euthanized 0.5, 1, 4, 8 or 24 h later. The spleens and lymph nodes were removed and individually analyzed for gene expression by RT-PCR. Statistically significant levels of splenic *fasL* ligand expression occurred 0.5-1 h after treatment with peak expression about seven-fold over control-treated mice. Peak *fas* expression was approximately three-fold over mice given isotype-matched control antibody and occurred 1 h after treatment with statistically significant, ($p < 0.001$), levels of expression from 1-8 h following anti-CD3 antibody treatment. The level of *bcl-2* expression remained relatively unchanged over the course of the experiment and was not statistically different than control-treated mice (Figure 14). To determine the source of the increased expression of *fas* and *fasL*, spleen or lymph node cells were recovered one h after anti-CD3- or hamster IgG antibody-treatment and labeled with anti-Thy1 antibody. The Thy1-labeled cells were sorted into positive and negative populations and examined for *fas*, *fasL* and *bcl-2* expression by RT-PCR. The source of the increased *fas* and *fasL* gene expression in mice given anti-CD3 antibody was Thy1$^+$ cells which showed a 16-fold increase in *fas* expression over untreated-unsorted splenocytes (Figure 15). *Fas* expression in the lymph nodes was less pronounced, most likely due to the difference in kinetics of anti-CD3 antibody-induced

activation or antibody trafficking. The expression of *fasL* was also increased about eight-fold in the Thy1$^+$ population (Figure 15).

To confirm that the increased *fas mRNA* expression in T cells led to increased expression of cell-surface Fas, splenocytes from mice injected with 20 μg of anti-CD3 antibody or hamster IgG antibody were examined 18 h after treatment. The splenocytes were incubated for two h then labeled with antibodies to CD4 and CD8 and cell surface Fas. The analysis gates were established to analyze Fas-expression on CD4$^+$ or CD8$^+$ cells. An increase in cell-surface Fas-expression on both CD4$^+$ and CD8$^+$ cells was detected (Figure 16). The data also revealed a background level of expression on hamster IgG treated lymphocytes indicating that Fas-expression was induced by hamster IgG *in vivo* or that lymphocytes have a background level of Fas-expression. These data support a model in which anti-CD3 antibody-induced apoptosis may be dependent on the *fas/fasL* pathway.

FIGURE 14. *In vivo* treatment with anti-CD3 antibody induces a rapid, transient increase in splenic *fas* and *fasL* but not *bcl-2* gene expression. Mice were injected with 20 μg of anti-CD3-or hamster IgG antibody. Mice were euthanized at the indicated time points and their spleens were removed. Following the isolation of total RNA from these tissues gene expression was examined by RT-PCR. Data points represent mean ± SEM values of a group of three mice, * $p < 0.05$, ** $p < 0.001$. These data are normalized to the endogenous standard HPRT which did not vary by more than three-fold throughout the experiment. The mean values of mice given anti-CD3 antibody were normalized to the mean expression of mice injected with isotype-matched hamster IgG control antibodies which were arbitrarily given a value of one.

Apoptosis Associated Gene Expression After Anti-CD3 Antibody Treatment

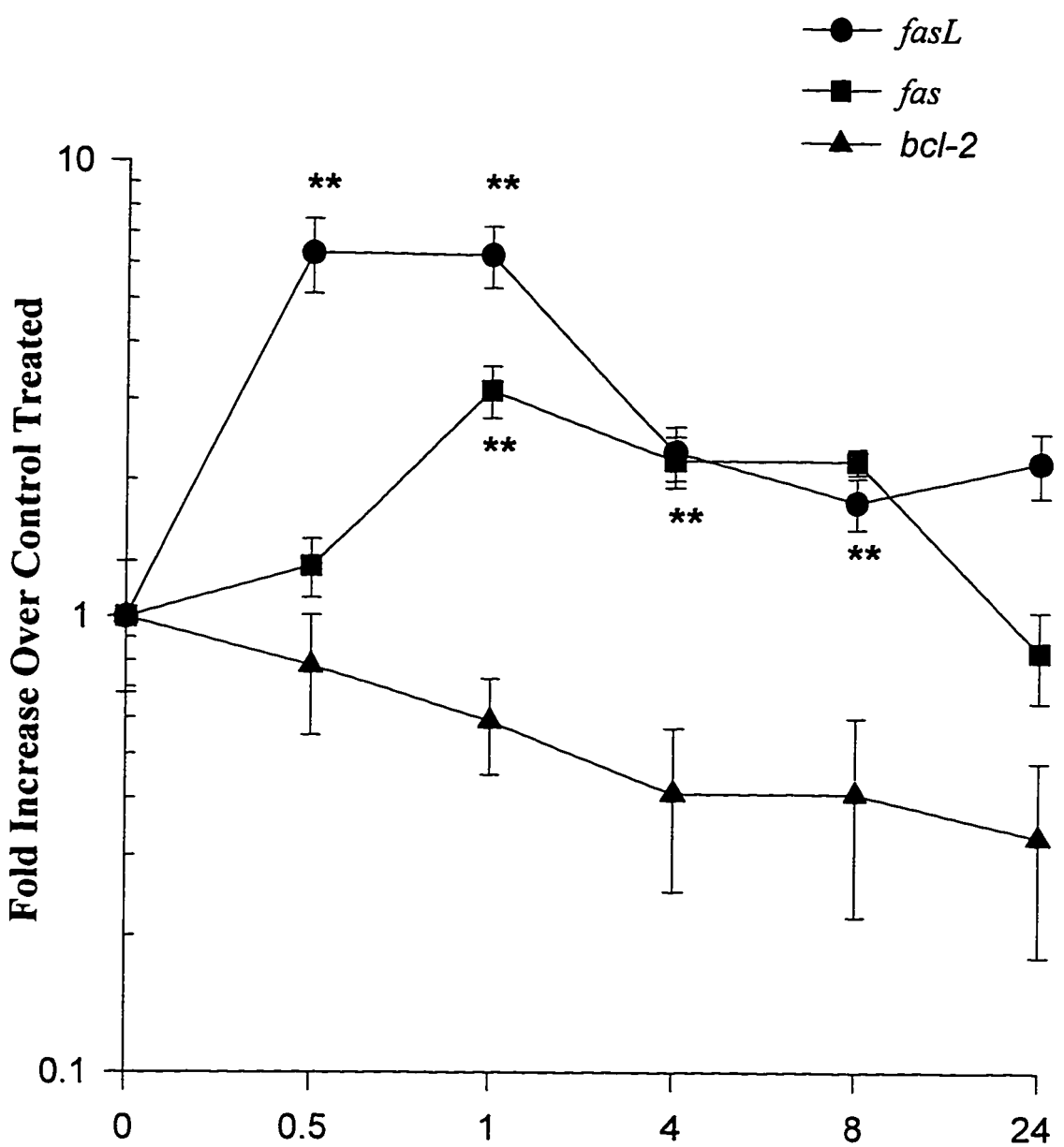


FIGURE 15. Thy1$^-$ cells are the source of increased *fas/fasL* expression. Mice were injected with 20 μg of anti-CD3-or hamster IgG antibody and were euthanized 1 h later. The spleens or brachial and axillary lymph node pairs were removed, stained with anti-Thy1 antibody and sorted with a Coulter EPICS Elite into positive and negative populations. The *fas* and *fasL* gene expression of pooled nodes or spleen was determined as described in the legend to Figure 14. The gene expression in the sorted cell populations is expressed as a fold-increase over hamster IgG treated-unsorted gene expression, arbitrarily assigned a value of one. These data are representative of three experiments for splenic gene expression and two experiments for lymph node gene expression.

Apoptotic Gene Expression in Splenic and Lymph Node Thy1$^+$ Cells

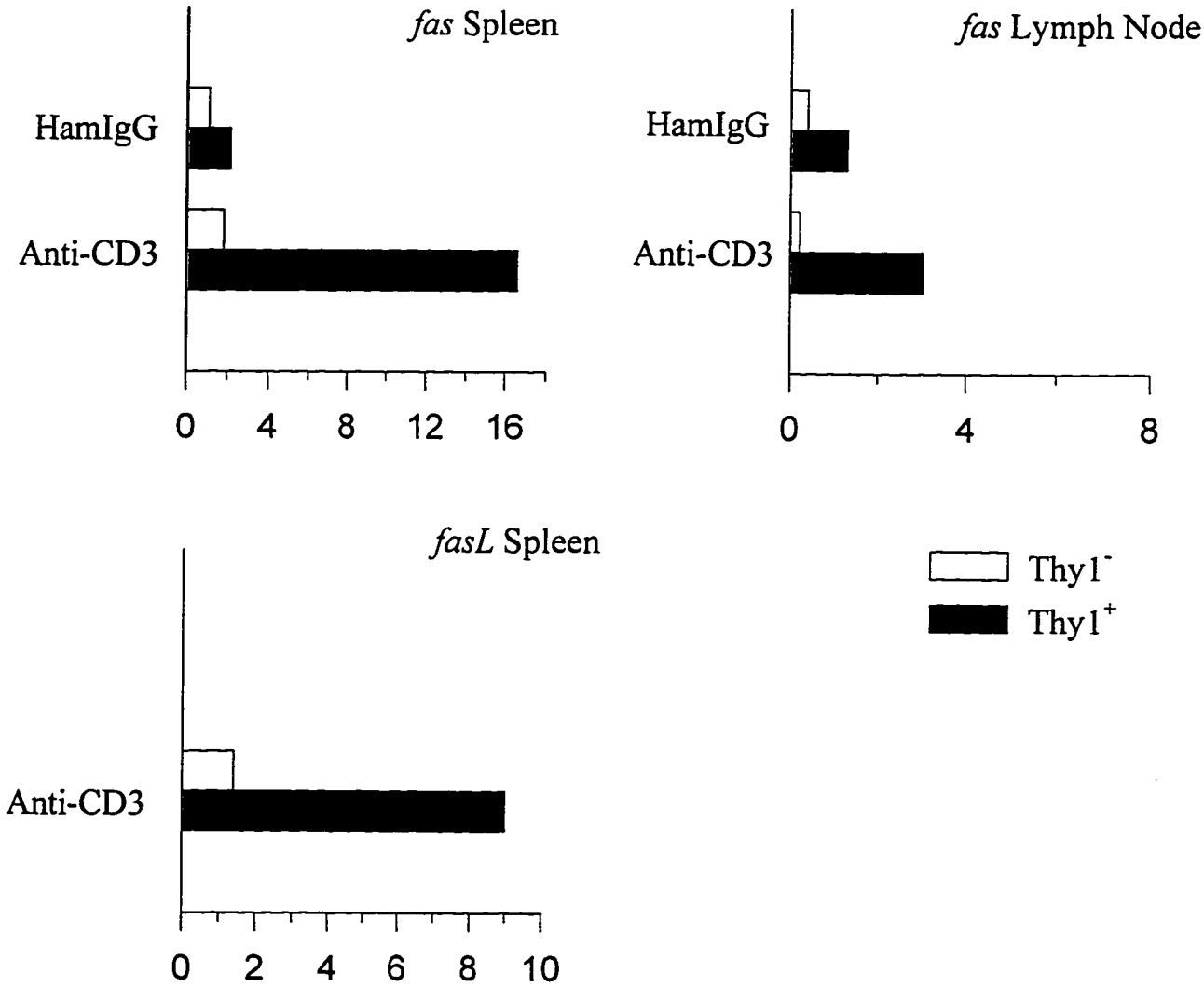
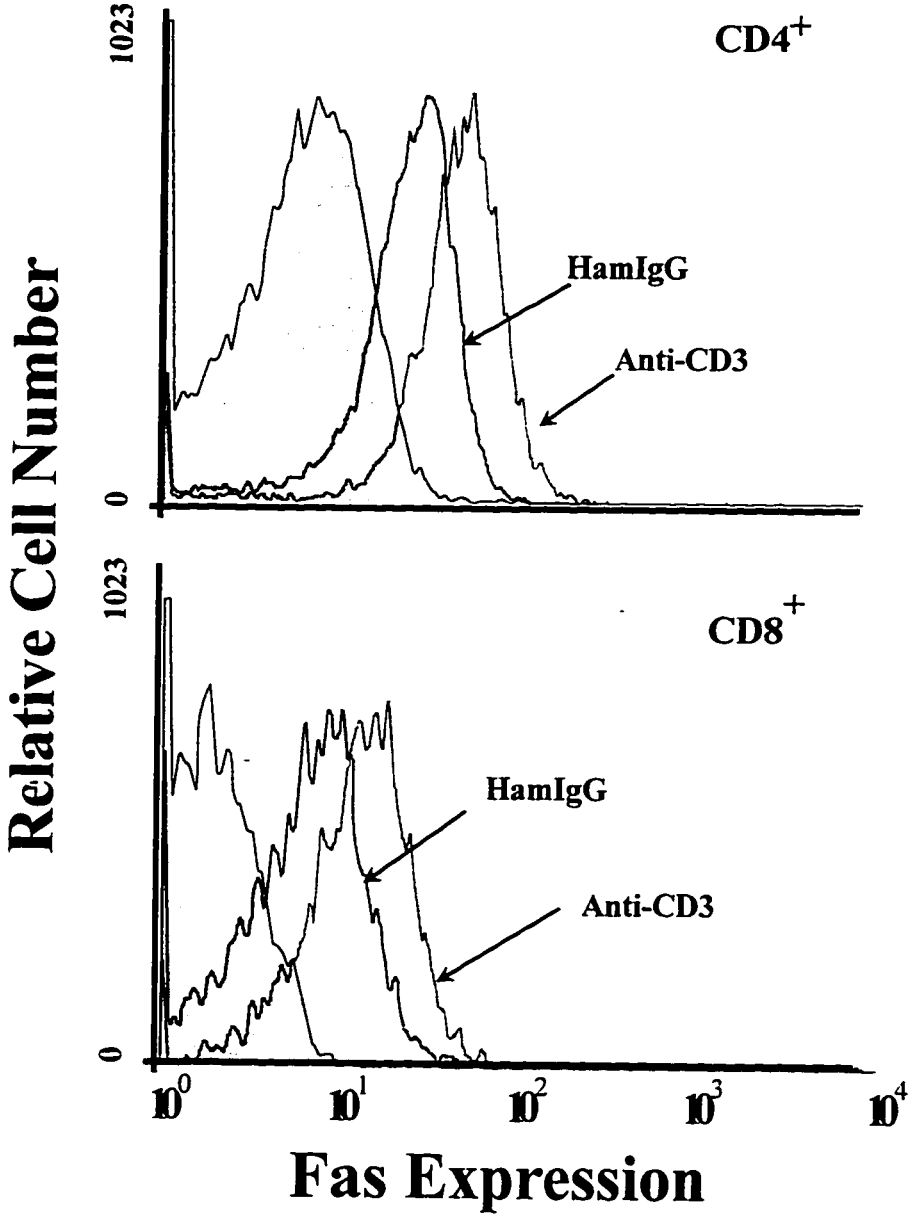


FIGURE 16. *In vivo* treatment with anti-CD3 antibody induces increased Fas expression on T cells. Mice were injected with 20 μg anti-CD3 or hamster IgG antibody and euthanized 18 h later. Cell suspensions of spleen were prepared and the cells were incubated for two h. Following incubation, the cells were surface labeled with antibodies against CD4 or CD8 and Fas then analyzed by FACS on a Coulter EPICS Elite. Gates were set on $CD4^+$ (top) or $CD8^+$ (bottom) cells and the Fas-fluorescent profiles were compared to hamster IgG-treated mice. The shaded histogram is the fluorescent profile of a hamster IgG isotype control for the hamster-anti-Fas antibody Jo2, on $CD4^+/CD8^+$ splenocytes from an anti-CD3 antibody-treated mouse. The histograms are representative of two experiments with three mice per group.

Anti-CD3 Antibody Treatment Increases Fas Expression on Splenic T cells



Part III. The induction of T cell associated cytokines by anti-CD3 antibody *in vivo* and their possible roles in apoptosis.

Induction of specific T cell-associated cytokines following in vivo administration of anti-CD3 antibody. The *in vivo* production of cytokines by T cells induced by anti-CD3 antibody has been reported (Scott *et al.*, 1990; Flamand *et al.*, 1990; Alegre *et al.*, 1991; Ferren *et al.*, 1990; Adkins and Hamilton.1992). To confirm the pattern of cytokine gene expression and to better define the kinetics of cytokine gene expression, mice were injected with 20 μg of anti-CD3-or hamster IgG antibody and euthanized 24, 8, 4, 1 or 0.5 h later. Total RNA from the spleen and brachial and axillary lymph node pairs were collected to examine the kinetics of splenic and lymph node gene expression for IL-2, IL-4, IL-10, and IFN-γ. Anti-CD3 antibody-treatment rapidly induced statistically significant, ($p < 0.05/0.001$), splenic gene expression of the T cell-associated cytokines IL-2 and IL-4 and IFN-γ but not IL-10. Significant splenic IL-2 and IFN-γ expression were induced as early as 30 minutes after treatment and the expression remained elevated to at least 24 h after treatment; but peak expression of IL-2 and IFN-γ occurred between 1-4 h after anti-CD3 antibody-treatment. IL-4 gene expression was rapidly induced, with peak expression 1 h following treatment, which rapidly decreased to control levels by 4 h after anti-CD3 antibody-treatment (Figure 17). Lymph node expression of IL-2 and IFN-γ were similar in magnitude but altered in kinetics with peak expression for IL-2 and IFN-γ 4 and 24 h following anti-CD3 antibody treatment, respectively (Figure 18). IL-4 expression in the lymph nodes was not as

pronounced as in the spleen, although an approximate ten-fold increase over control-treated mice could be detected at all time points through eight h following treatment (Figure 18).

Since IL-2 and IL-4 have important roles in T cell activation and the prevention of anergy, it was important to determine if T cells were the source of these cytokines. Mice were injected with 20 μg of anti-CD3-or hamster IgG antibody and euthanized 90 minutes following treatment. The splenocytes were surfaced labeled with anti-Thy1 antibody. The labeled splenocytes were sorted into positive and negative populations, total RNA was isolated and IL-2 and IL-4 mRNA were analyzed by RT-PCR. The source of anti-CD3 antibody induced IL-2 and IL-4 mRNA was Thy 1^- cells, which expressed a more than 280 fold increase in IL-4 mRNA and a nearly 400 fold increase in IL-2 mRNA over unsorted control-treated splenocytes (Figure 19).

FIGURE 17. *In vivo* administration of anti-CD3 antibody initiates rapid splenic expression of T cell derived cytokines. Mice were injected with 20 μg of anti-CD3 or hamster IgG antibody 24, 8, 4, 1, or 0.5 h before being euthanized. Total RNA was isolated and gene expression was examined by RT-PCR as described in the Materials and Methods. Data are presented as the mean ± SEM for three mice per time point. The data are normalized to the endogenous standard HPRT which varied less than three-fold throughout the experiment. The mean values of mice given anti-CD3 antibody were normalized to the mean gene expression of mice injected with isotype matched hamster IgG control antibodies, which were arbitrarily given a value of one. Statistically significant elevations of cytokines in anti-CD3 antibody-treated mice over hamster IgG antibody-treated mice are as follows: IFN-γ and IL-2 $p < 0.05$, at 0.5 and 24 h $p < 0.001$ at 1,4 and 8 h, IL-4 $p < 0.05$, at 0.5 and 8 h. $p < 0.001$ at 1 h.

Splenic Gene Expression Following *In Vivo* Treatment with Anti-CD3 Antibody

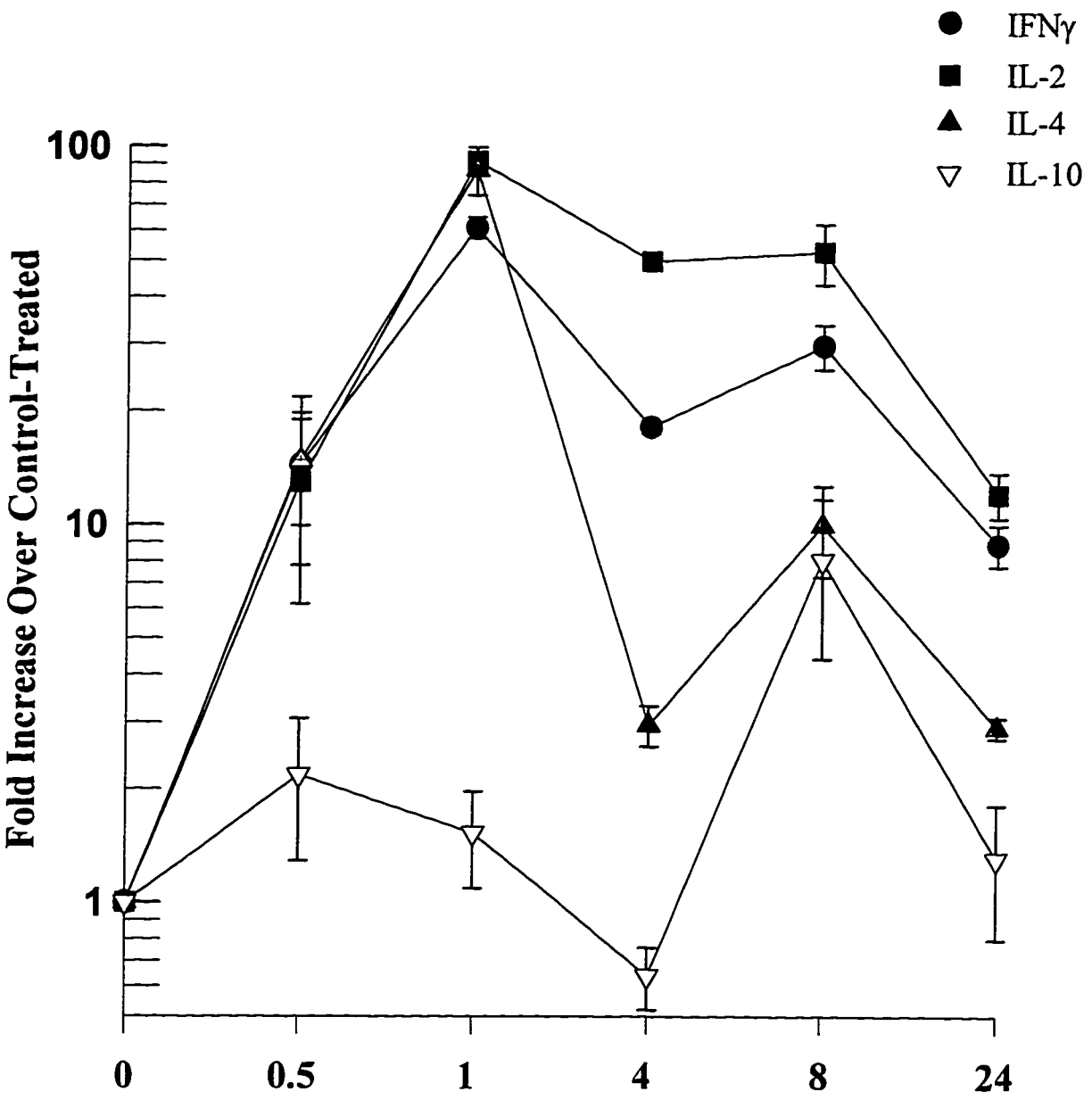


FIGURE 18. *In vivo* administration of anti-CD3 antibody initiates rapid lymph node expression of T cell derived cytokines. Mice were injected and gene expression examined as described in the legend to Figure 17. Data are presented as the mean for pooled brachial and axillary lymph nodes from three mice per time point. The data are normalized to the endogenous standard HPRT which varied less than three-fold throughout the experiment. The mean values of mice given anti-CD3 antibody were normalized to the mean expression of mice injected with isotype matched hamster IgG control antibodies which were arbitrarily given a value of one.

Lymph Node Gene Expression Following *In Vivo* Treatment With Anti-CD3 Antibody

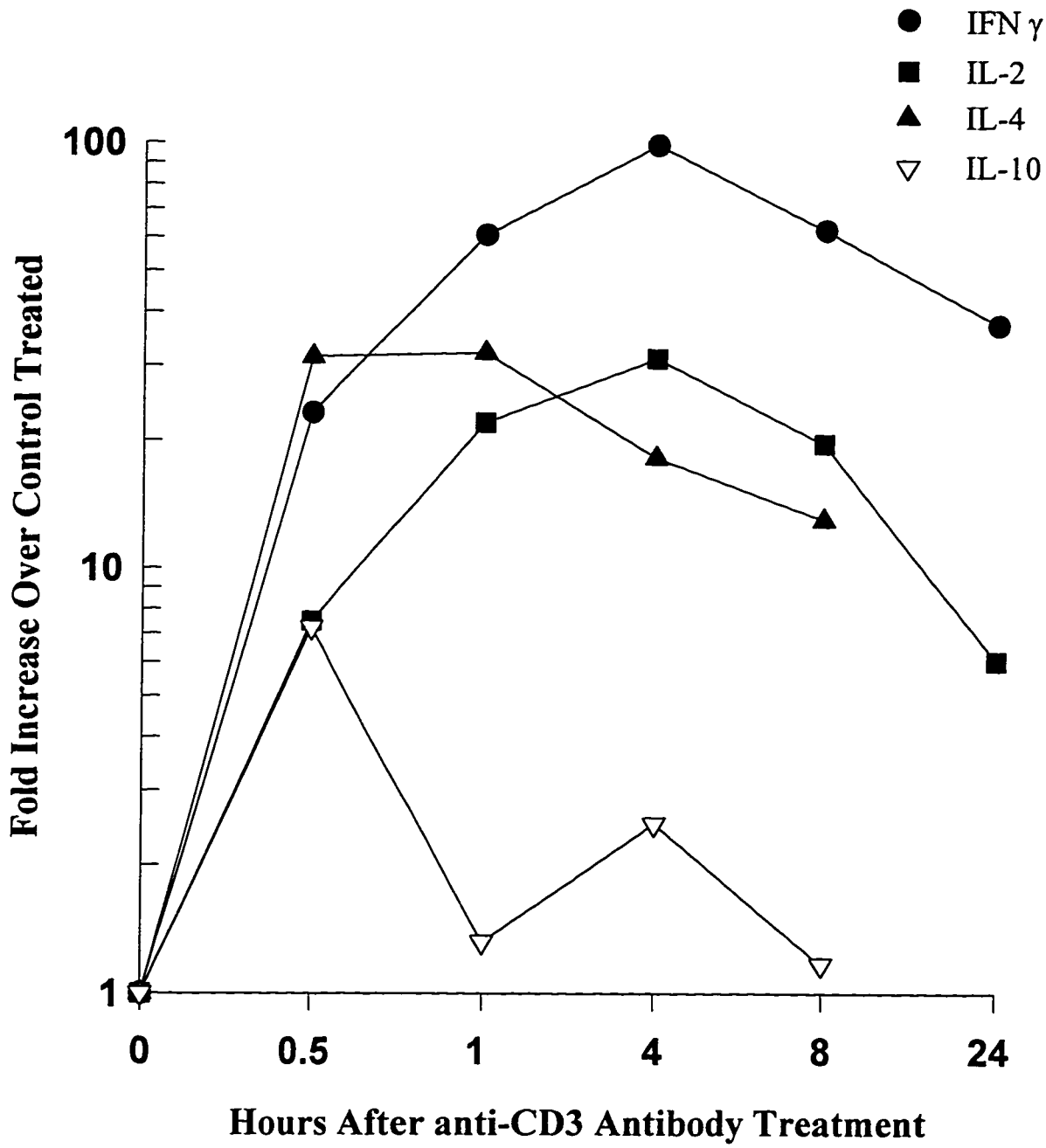
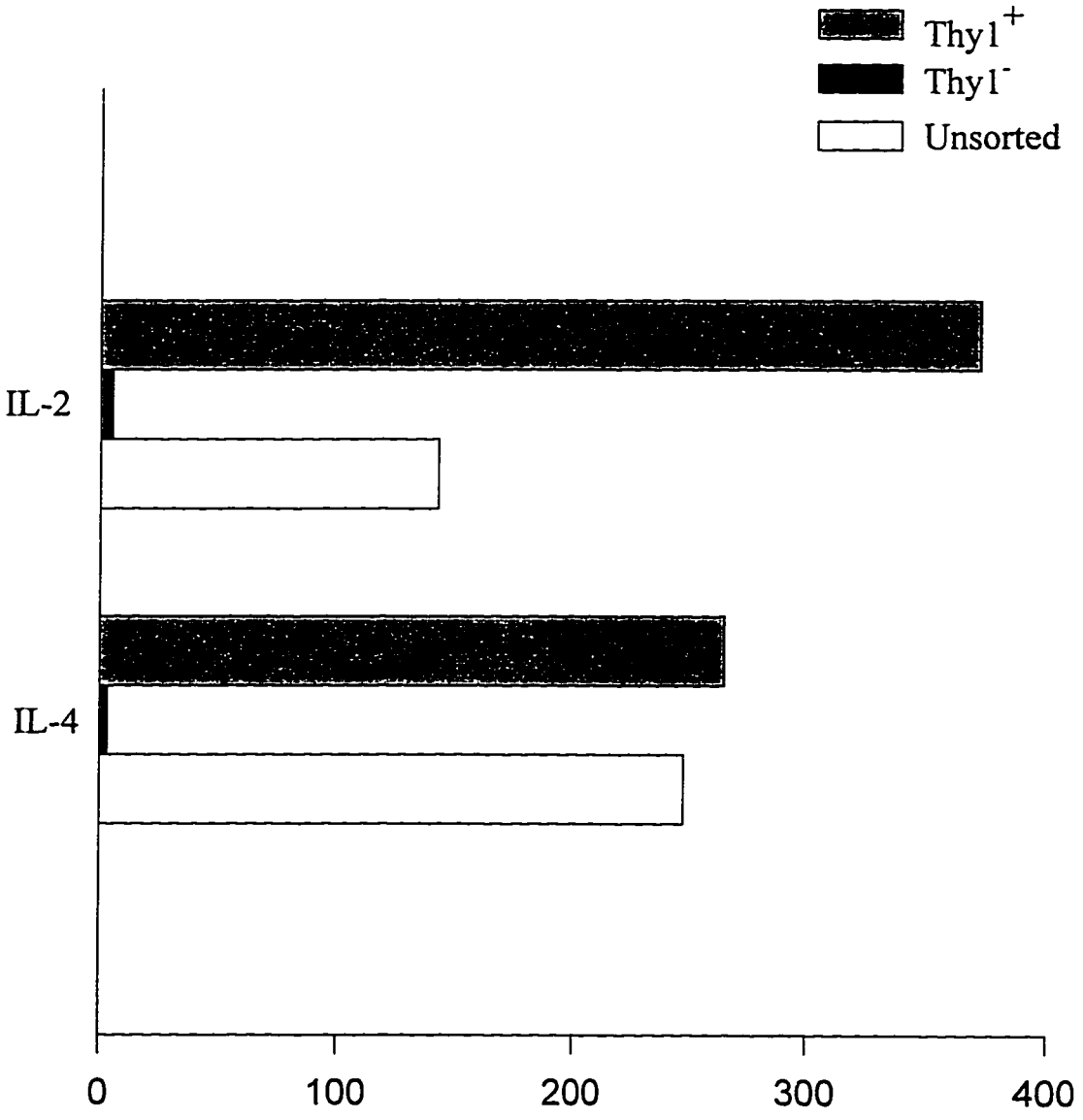


Figure 19. Thy1$^+$ spleen cells are the source of splenic IL-2 and IL-4 mRNA following treatment with anti-CD3 antibody. BALB/c mice were injected with 20 μg anti-CD3 or hamster IgG antibody and euthanized 90 minutes later. Splenocytes were labeled with the T cell marker Thy1 and sorted into positive and negative populations. Total cellular RNA was extracted from the sorted and unsorted populations and equivalent amounts of RNA were analyzed by RT-PCR. The resulting expression of IL-2 and IL-4 mRNA was normalized to the housekeeping gene HPRT and expressed as a fold-increase over hamster IgG control-treated unsorted splenocytes which were arbitrarily assigned a value of one. Each data point represents pooled splenocytes with five mice per group. The results are representative of three experiments.

Thy1$^+$ Splenocytes Are The Source of Anti-CD3 Antibody-Induced IL-2 and IL-4 mRNA



A specialized CD4$^+$NK1.1$^+$ subset produces more IL-4 than CD4$^+$ cells in response to in vivo treatment with anti-CD3 antibody and is more resistant to apoptosis. It was recently reported that *in vivo* treatment with low doses of anti-CD3 antibody induces IL-4 gene expression exclusively in a specialized T cell subset (Yoshimoto and Paul.1994). This differential IL-4 expression raised the possibility that anti-CD3 antibody-induced apoptosis occurred in the CD4$^+$ NK1.1$^-$ T cell subset that was not producing IL-4. To determine whether CD4$^+$ NK1.1$^+$ T cells were producing IL-4 in response to anti-CD3 antibody-treatment while other T cell subsets were subsequently undergoing apoptosis, or whether these events occurred in both T cell subsets, gene expression and apoptosis of CD4$^+$ NK1.1$^+$ cells and CD$^+$ NK1.1$^-$ were compared. Mice were treated with 20 μg (high dose) or 0.1 μg (low dose) of anti-CD3 or hamster IgG antibody and euthanized one h later. Splenic IL-4 gene expression was analyzed using the RT-PCR assay. The data show that anti-CD3 antibody-treatment induced almost a 100-fold increase with low dose, and an almost 400-fold increase in IL-4 mRNA expression at the high dose of anti-CD3 antibody treatment in CD4$^+$ NK1.1$^+$ cells over control-treated unsorted splenocytes. These results confirm and expand the previous findings that CD4$^+$ NK1.1$^+$ cells produce the majority of IL-4 in response to anti-CD3 antibody treatment but that CD4$^+$ NK1.1$^-$ cells also produce IL-4 which becomes more significant as the dose of anti-CD3 antibody increases (Figure 20).

To determine if IL-4-producing CD4$^+$ NK1.1$^+$ cells were resistant to anti-CD3 antibody-induced apoptosis mice were treated with 20 μg of anti-CD3 or hamster IgG antibody and euthanized 48 h later. The splenocytes were labeled with antibodies to CD4 and NK1.1; DNA fragments were labeled and analyzed by the FLANUL assay. These data

show that anti-CD3 antibody induces a statistically significant, ($p < 0.001$), four-fold increase in the percentage of $CD4^+$ $NK1.1^-$ UTP^+ cells from 3.5% in hamster IgG-treated mice to 12.6% in anti-CD3 antibody-treated mice. The $CD4^+$ $NK1.1^-$ cells are more resistant to anti-CD3 antibody-induced apoptosis because the proportion of these cells increases in the remaining population of $CD4^+$ cells following *in vivo* treatment with anti-CD3 antibody. As a percent of the total $CD4^+$ cells, nonapoptotic $CD4^+$ $NK1.1^-$ UTP^- cells increased from 0.7% in control antibody-treated mice to 16.8% in anti-CD3 antibody-treated mice. These statistically significant, ($p < 0.05$), results suggest that as $CD4^+$ cells were undergoing apoptosis, the proportion of $CD4^+$ $NK1.1^-$ cells rose due to their increased resistance to anti-CD3 antibody-induced apoptosis. These data also indicate that the $CD4^+$ $NK1.1^-$ cells undergo a low level anti-CD3 antibody-induced apoptosis as the proportion of these cells incorporating UTP increases from 0.1% in control antibody-treated animals to 4.4% in anti-CD3 antibody-treated animals (Table 6). Collectively these experiments suggest that apoptosis and IL-4 production are differentially regulated. $CD4^+$ $NK1.1^-$ cells produce IL-4 and are more resistant to anti-CD3 antibody-induced apoptosis while $CD4^+$ $NK1.1^-$ cells produce less IL-4 and are highly susceptible to anti-CD3 antibody-induced apoptosis.

FIGURE 20. A specialized subset of cells produces the majority of IL-4 following *in vivo* treatment with anti-CD3 antibody. C57BL/6 mice were treated with 0.1 μg (low dose) or 20 μg (high dose) of anti-CD3-or hamster IgG antibody and euthanized 1 h later. Cell suspensions were prepared from pooled spleens from each treatment group were surface labeled with antibodies to CD4 and NK1.1. The cells were sorted with a Coulter EPICS Elite into $CD4^{-}NK1.1^{-}$ and $CD4^{+}NK1.1^{-}$ subpopulations. Total cellular RNA was extracted from the sorted and unsorted populations and equivalent amounts of RNA were analyzed by RT-PCR. The resulting expression of IL-4 mRNA was normalized to the housekeeping gene HPRT and then expressed as a fold-increase over hamster IgG control-treated unsorted splenocytes which were arbitrarily assigned a value of one. Each data point represents pooled splenocytes with five mice per group.

$CD4^+NK1.1^+$ Splenocytes Express The Majority of Anti-CD3 Antibody-Induced IL-4

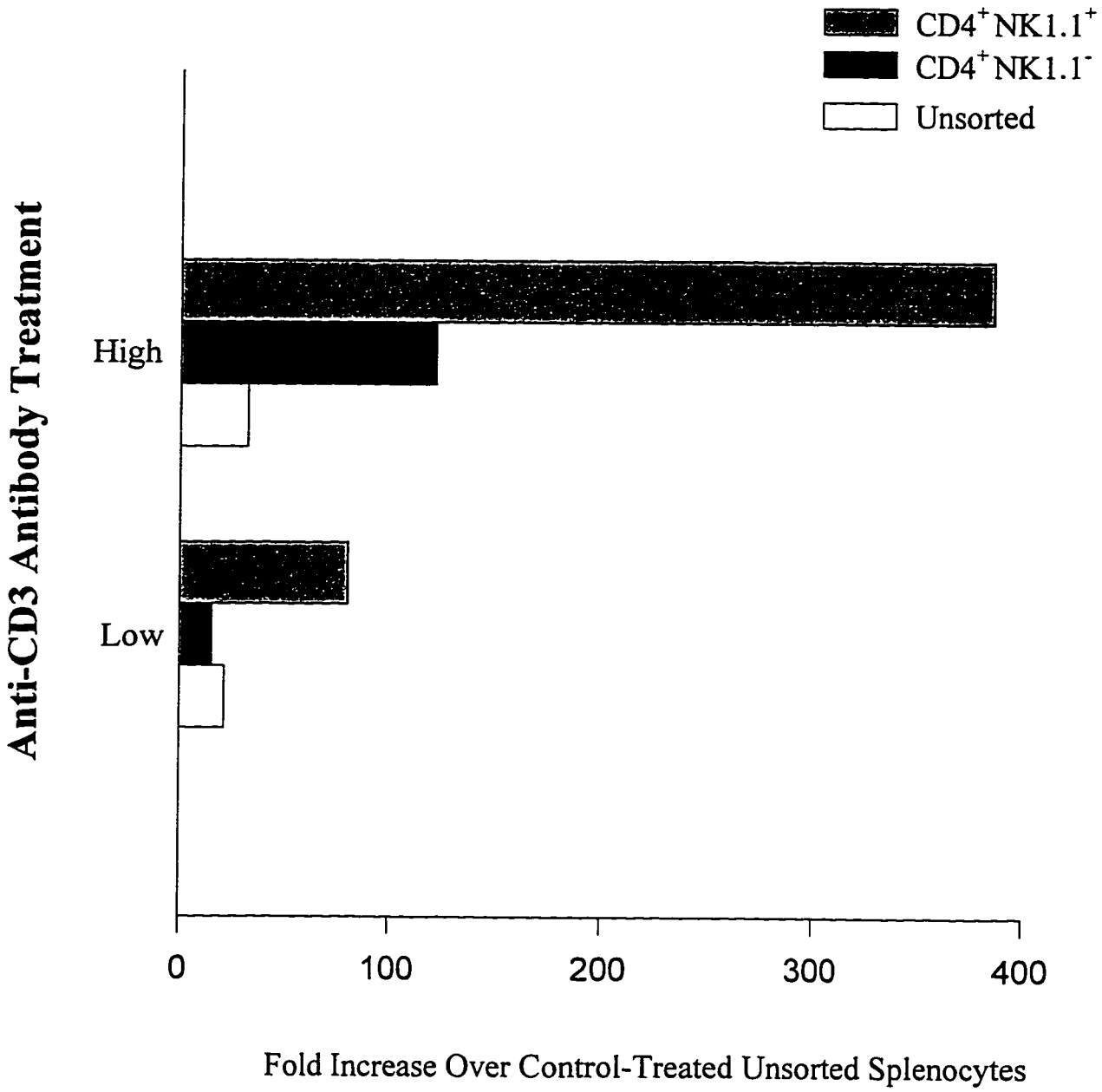


Table 6. Quantitation of Apoptotic Splenic $CD4^+$ T Cells 48 Hours Following *In Vivo* Administration of Anti-CD3 Antibody.

GROUP[a]	Phenotype[b]	Mean ± SEM%[c]	
		UTP^+	UTP^-
hamster IgG	$NK\ 1.1^-$	3.5 ± 0.1	
	$NK\ 1.1^+$	0.1 ± 0.1	0.7 ± 0.2
anti-CD3	$NK\ 1.1^-$	12.6 ± 0.6**	
	$NK\ 1.1^+$	4.4 ± 1.1*	16.8 ± 4.7*

[a] C57BL/6 mice (3 per group) were given iv injections of 20 μg of hamster IgG or anti-CD3 antibody and euthanized 48 h later.

[b] Splenocytes were stained with anti-CD4-Cy5 and anti-NK1.1-FITC and free 3' ends of DNA were labeled with biotinylated dUTP and SA-PE as described in the Materials and Methods. Gates were set on $CD4^+$ cells and were examined for NK1.1 staining.

[c] $CD4^+$ $NK1.1^-$ and $CD4^+$ $NK1.1^+$ cells were examined for UTP incorporation and the data reflects the mean ± SEM for cells staining with UTP (apoptotic) or without UTP (non-apoptotic), * $p < 0.05$, ** $p < 0.001$ (when compared to hamster IgG treated phenotype).

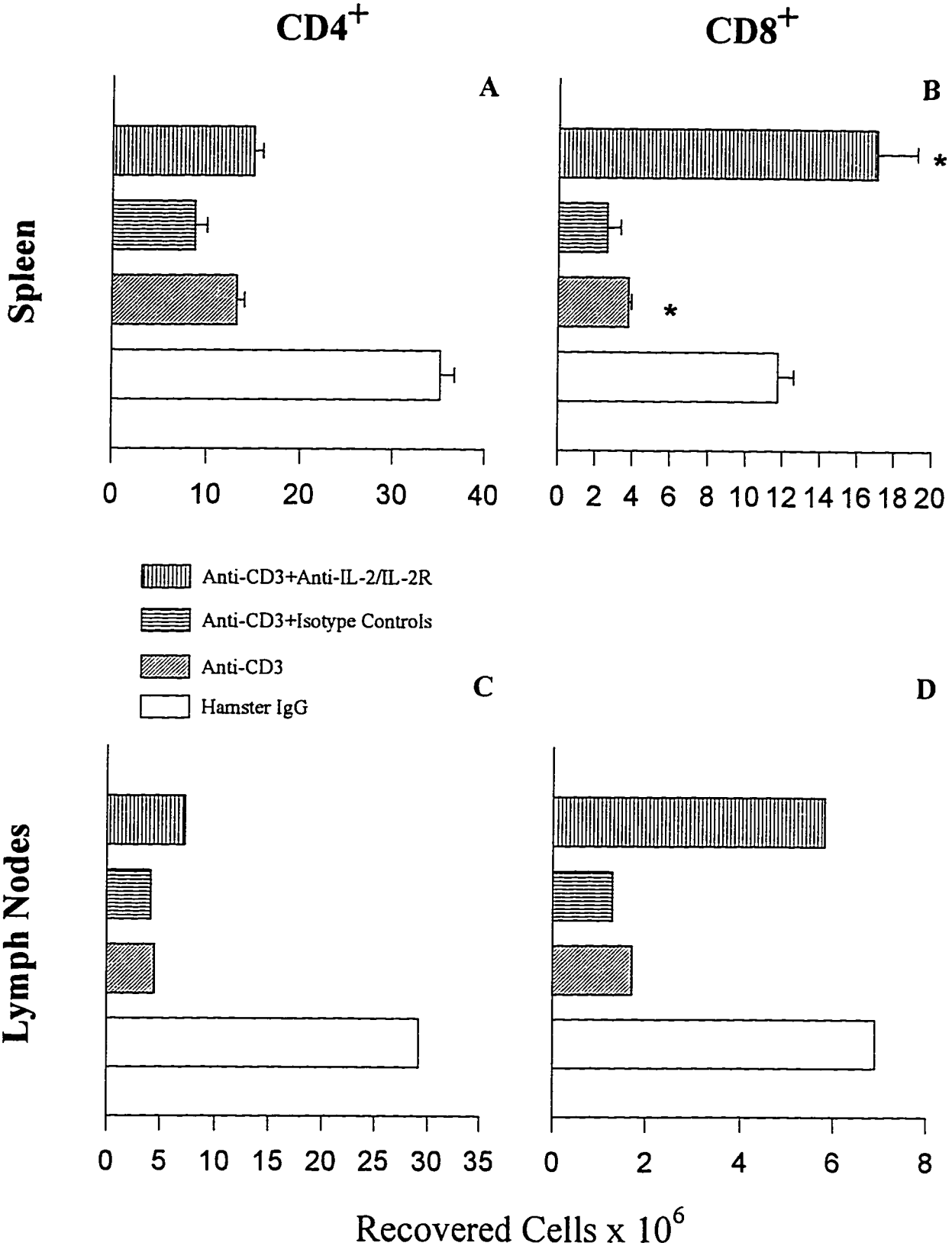
The in vivo administration of exogenous cytokines cannot rescue T cells from anti-CD3 antibody-induced apoptosis. The stimulation of T cells *in vitro*, in the absence of appropriate costimulation, leads to a short burst of IL-2 production which is not sufficient to stimulate T cells to proliferate and differentiate to cytokine-producing effector cells (June *et al.*, 1987). Therefore, a major function of costimulation may be maintaining IL-2 production. If TCR signaling occurs in the absence of a second costimulatory signal, the lack of sustained IL-2 production will lead to the induction of anergy, and *in vivo* may lead to activation-induced apoptosis (Mueller *et al.*, 1989; Fraser and Weiss.1992; Seder *et al.*, 1994; Norton *et al.*, 1992). In a process termed "propriocidal regulation" treatment of T cells with IL-2 sensitizes these cells to apoptosis upon subsequent activation with anti-CD3 antibody *in vitro* (Boehme and Lenardo.1993). Conversely, the provision of exogenous IL-2 prior to or after anti-CD3 antibody-treatment may activate T cells and or induce gene expression which diverts apoptosis. To determine the fate of anti-CD3 antibody-stimulated T cells provided exogenous IL-2, BALB/c mice were given IL-2/anti-IL-2 complexes either shortly before or following anti-CD3 antibody-treatment. IL-2/anti-IL-2 cytokine complexes are long-lived *in vivo*. The half-life of the IL-2 complex was shown to be days, while that of recombinant IL-2 alone was found to be only seven minutes in humans (Finkelman *et al.*, 1993; Lotze *et al.*, 1985). Therefore, the administration of IL-2 as a complex increases the half-life of the cytokine. The administration of two doses of IL-2/anti-IL-2 (5 μg/25 μg) complex, one dose on D 0, 1 h before administration of 20 μg of anti-CD3 antibody and a second dose 24 h after anti-CD3 antibody-treatment did not divert anti-CD3 antibody-induced apoptosis. When the IL-2 complexes were given sequentially on D+1 and D+3

following anti-CD3 antibody-treatment, the mice died within 24 h. A single dose of IL-2 complex given either 1 hour before or 24 h after anti-CD3 antibody-treatment was not lethal nor did it rescue T cells from depletion (data not shown). These data argue against a role for IL-2 in preventing anti-CD3 antibody-induced apoptosis. These results do suggest that IL-2 may sensitize T cells to activation-induced cell death as previously hypothesized or that it may hyper-activate T cells to produce inflammatory cytokines which cause death in the mouse.

To test the hypothesis that IL-2 may sensitize T cells for anti-CD3 antibody-induced apoptosis, the utilization of endogenous IL-2 produced after treatment with anti-CD3 antibody was blocked with a combination of anti-IL-2 and anti-IL-2R antibodies. A combination of anti-IL-2 and anti-IL-2R antibodies was given to mice 12 h prior to administration of 20 μg of hamster IgG or anti-CD3 antibody. Splenic and lymph node T cell depletion was measured four days after anti-CD3 antibody-treatment. These data show a statistically significant, ($p < 0.001$), rescue of $CD8^+$ splenic and lymph node T cells but not $CD4^+$ cells, from anti-CD3 antibody-induced depletion, when pretreated with of anti-IL-2/anti-IL-2R antibody. The experiments did show a partial rescue from depletion of splenic $CD4^+$ cells, but this rescue was not significant, ($p > 0.05$), and is clearly different than $CD8^+$ cell rescue (Figure 21). These results suggest that the anti-CD3 antibody-induced early expression of IL-2 (Figures 17-19) sensitizes $CD8^+$ T cells to deletion and that $CD4^+$ T cells undergo deletion independently of the IL-2 pathway.

Figure 21. The IL-2/IL-2R pathway is not required for the anti-CD3 antibody-induced deletion of $CD4^+$ cells, but is required for CD8 peripheral T cells. BALB/c mice were injected with 3 mg of anti-IL-2 and 3 mg of anti-IL-2R antibody 12 h prior to treatment with 20 μg hamster IgG or anti-CD3 antibody. The mice were euthanized four days later and the spleens and lymph nodes were removed. Cell preparations from these tissues were stained with antibodies to the T cell surface markers CD4 and CD8. The percentage of cells staining positive for either CD4 or CD8 was multiplied by the calculated total number of recovered cells. The number of recovered splenic: A) $CD4^+$ B) $CD8^+$ cells, or lymph node: C) $CD4^+$ or D) $CD8^+$ cells, are shown. These data are representative of three experiments. Lymph node data represents $CD4^+$ and $CD8^+$ cells from three mice, four nodes per animal pooled, spleen cell data represent the mean ± SEM (n=3), * $p < 0.05$ ** $p < 0.001$.

The IL-2/IL-2R Pathway is Required For The Anti-CD3 Antibody-[111] Induced Deletion of $CD8^+$ But Not $CD4^+$ Peripheral T cells



Several *in vivo* studies confirm that T helper subsets have differential responses to tolerance induction. IL-4 producing (Th2) cells are more resistant to tolerization by anti-CD3 antibody than IL-2 producing (Th1) cells *in vitro* (Seder *et al.*, 1994; Lichtman *et al.*, 1988; Williams *et al.*, 1992). The basis for this difference is unknown but could be due to the production and utilization of IL-4 as a costimulatory signal by Th2 cells, differential requirements between Th1 and Th2 cells for costimulation, or the requirement for IL-2 accessory signals to prevent anergy in Th1 cells (Burstein and Abbas.1993). If the observed *in vitro* anergy is a step towards anti-CD3 antibody-mediated apoptosis then the provision of accessory signaling with IL-4 may prevent a homologous event that leads to apoptosis *in vivo*. Naive T cells *in vivo* can be greatly influenced by exogenous cytokines and their development influenced towards a Th1 or Th2 pathway by the cross-regulatory cytokines IL-4 and IFN-γ (Williams *et al.*, 1992). In an attempt to influence the development of naive T cells through the administration of exogenous IL-4 complexes, mice were given IL-4 complexes six h prior to administration of hamster IgG or anti-CD3 antibody. Four days later the mice were euthanized and peripheral splenic and lymph node CD4 and CD8 T cells were counted. Administration of IL-4 complexes were unable to prevent the loss of either $CD4^+$ or $CD8^+$ splenic or lymph node T cells, but mice given IL-4 complexes alone had increased numbers of peripheral $CD4^+$ and $CD8^+$ cells. Thus, the IL-4 complexes were able to induce T cell proliferation as previously reported (Finkekman *et al.*, 1993), but were unable to prevent the anti-CD3 antibody-induced loss of either CD4 or CD8 T cells (data not shown). These results are consistent with the previous finding that there is no difference in

anti-CD3 antibody-induced apoptosis between Th1 and Th2 clones *in vitro* (Russell *et al.*, 1992).

IL-1 was also tested for its ability to prevent anti-CD3 antibody-induced T cell depletion. IL-1 has direct effects on T cells, it is comitogenic and increases the endogenous production of IFN-γ, CSFs, IL-4, IL-5, and IL-2 (Oppenheim *et al.*, 1991; Arai *et al.*, 1990). IL-1 indirectly effects T cells through its mitogenic effects on macrophages, B cells and other cell types. This indirect effect could divert T cell apoptosis through the up-regulation and interaction of secondary receptor ligand pairs, through accessory signals mediated by cytokine secretion, or through FcR interactions on the APC (McArthur and Raulet.1993; Oppenheim *et al.*, 1991; Arai *et al.*, 1990). Therefore, mice were given exogenous IL-1α either one day before or with anti-CD3 antibody or hamster IgG antibody, and four days later splenic CD4 and CD8 cells were counted. Mice treated with IL-1 did not have significantly more $CD4^+$ or $CD8^+$ splenic or lymph node T cells than mice treated with anti-CD3 antibody alone (data not shown). The 2 μg dose of exogenous IL-1 given one day before anti-CD3 antibody appeared to be marginally (but not statistically, $p > 0.05$) effective in reducing the loss of splenic $CD8^+$ but not $CD4^+$ T cells. This suggests that higher doses of IL-1 may be more effective in preventing T cell depletion. Also, the pyrogenic activity, that is mediated by larger doses of IL-1, has detrimental effects on mice (Dr. Ruth Neta, AFRRI, personal communication), and therefore additional studies were not undertaken. These results suggest that signals in addition to IL-1 are required *in vivo* to rescue T cells from anti-CD3 antibody-induced apoptosis. Further studies to directly address the role of B7-dependent costimulation in anti-CD3 antibody-induced apoptosis were undertaken and are presented in Part IV.

Part IV. The role of costimulation in anti-CD3 antibody-induced apoptosis and cytokine gene expression.

The role of endogenous B7-dependent T cell costimulation for cytokine expression following anti-CD3 antibody-mediated T cell signaling. The induction of IL-2 and IFN-γ by anti-CD3 antibody *in vitro* is dependent on B7-dependent costimulation, while production of IL-4 is independent of B7-costimulation (Jenkins *et al.*, 1990; Tamura *et al.*, 1993; Williams *et al.*, 1990). *In vivo,* the induction of Th1 cytokines by alloantigen is dependent on costimulation, but IL-4 production was shown to be costimulation independent (Sayegh *et al.*, 1995). Therefore, it was hypothesized that endogenously expressed costimulatory signals, in particular B7-dependent costimulation, may be required for T cell activation leading to cytokine production or apoptosis. To determine whether the anti-CD3 antibody-induced IL-4 response was B7-dependent, BALB/c mice were given CTLA4-Ig at two time points in various doses 24 h before and concurrently with 20 μg of hamster IgG or anti-CD3 antibody. The ability of CTLA4-Ig to block anti-CD3 antibody-induced IL-4 mRNA expression 90 minutes after treatment was measured by RT-PCR. At high doses of anti-CD3 antibody treatment (20 μg) the addition of mCTLA4-Ig from 50 μg to 300 μg per animal did not inhibit the production of IL-4 (Figure 22). There was no statistical difference, ($p > 0.05$), between mice given anti-CD3 antibody with CTLA4-Ig or L6 at any of the tested doses.

Recently, it was demonstrated that CTLA4-Ig could block low dose anti-CD3 antibody-induced IL-4 production (Yoshimoto and Paul.1994). Therefore, mice were treated with 200 μg of mCTLA4-Ig or the control fusion protein L6 in 100 μg doses 24 h and 1 h

before low dose (0.1/2 μg) or high dose (20 μg) anti-CD3 antibody treatment. The splenic IL-4 mRNA expression was determined 90 minutes following anti-CD3 antibody treatment by RT-PCR. These data show that mCTLA4-Ig, but not the control fusion protein L6, significantly reduced IL-4 mRNA at the low dose (0.1 μg) but not the higher doses of anti-CD3 antibody treatment (top of Figure 23). To determine if the inhibition of IL-4 mRNA expression by blocking B7-dependent costimulation also inhibited IL-4 protein expression, mice were treated as above at both the low and high doses of anti-CD3 antibody and splenocyte IL-4 protein production was measured in the ELISPOT assay. As with IL-4 gene expression, CTLA4-Ig was unable to inhibit IL-4 production following treatment with high doses of anti-CD3 antibody but was able to inhibit IL-4 protein production after treatment with lower doses of anti-CD3 antibody (bottom of Figure 23). The inhibition of low-dose anti-CD3 antibody-induced IL-4 production by CTLA4-Ig was statistically significant, $p < 0.001$. These data support a model of differential signaling in T cells. This signaling in the form of a strong signal one (high dose anti-CD3 antibody) can induce T cells to produce cytokines, in particular IL-4 independently of B7 costimulation but when signal one is not sufficiently strong (*i.e.*, low dose anti-CD3 antibody treatment) endogenous B7-dependent costimulation is required for full IL-4 expression.

Figure 22. Induction of IL-4 mRNA by high dose anti-CD3 antibody treatment is not blocked by CTLA4-Ig. BALB/c mice were injected with 50/100/200/300 μg of the chimeric protein CTLA4-Ig or the control protein L6. One-half the dose was given 24 h prior to anti-CD3 antibody-treatment and the second half concurrently with 20 μg hamster IgG or anti-CD3 antibody. Mice were euthanized 90 minutes later and total splenic RNA was isolated, and IL-4 mRNA expression determined by RT-PCR. The expression of IL-4 mRNA was normalized to the housekeeping gene HPRT and expressed as a fold-increase over hamster IgG control-treated splenocytes which were arbitrarily assigned a value of one. Each data point represents the mean ± SEM for three mice per group.

The Increase in Splenic IL-4 mRNA By High Dose Anti-CD3 Antibody Cannot Be Blocked By CTLA4-Ig

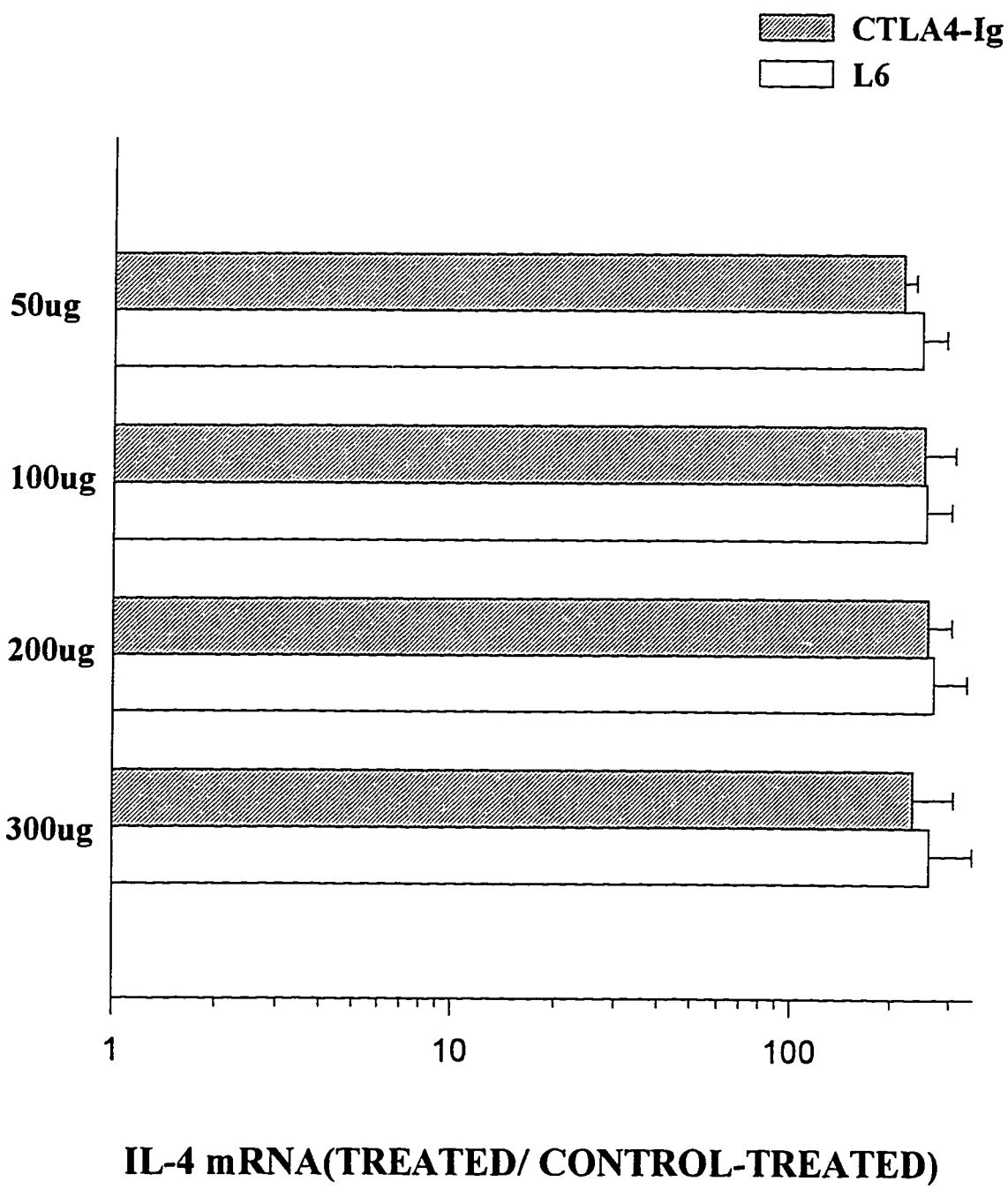
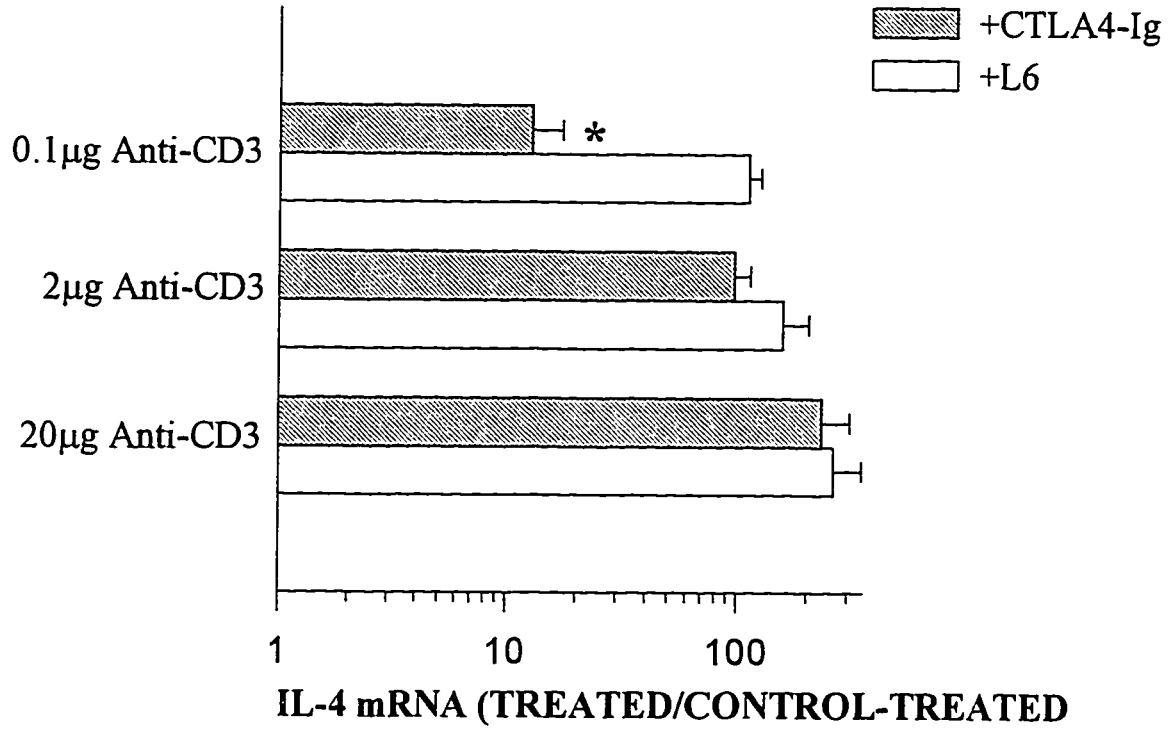
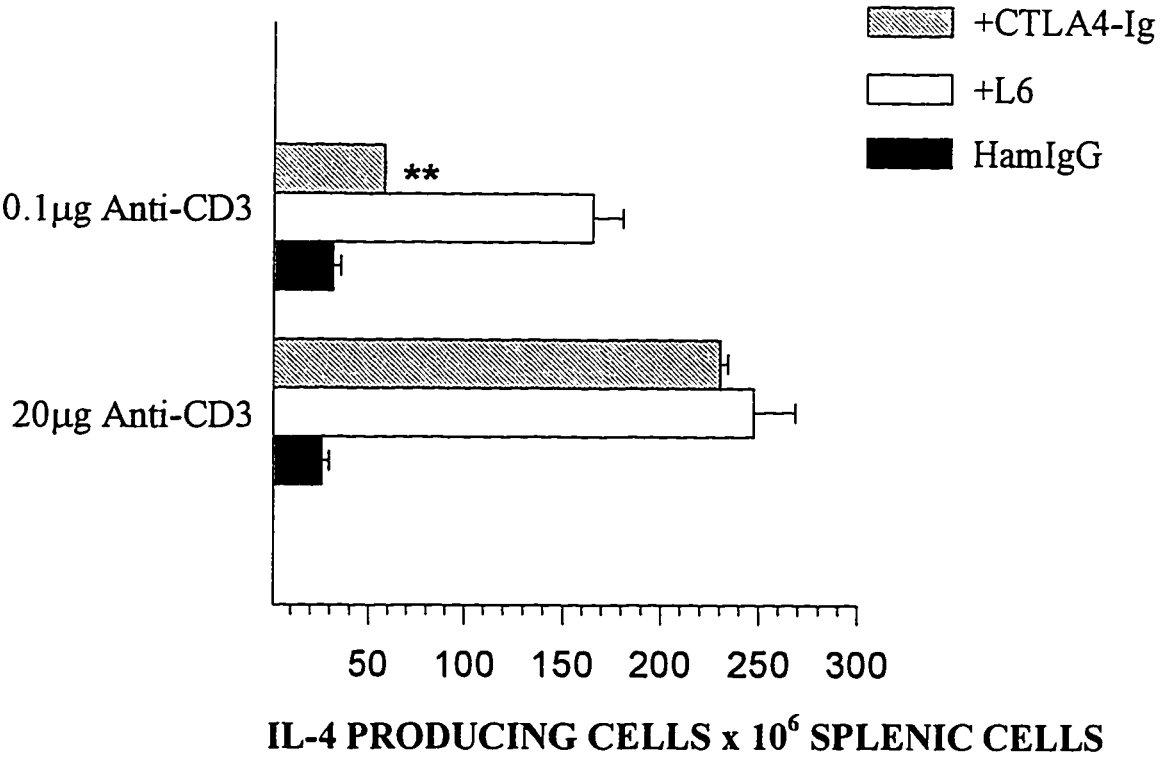


FIGURE 23. *In vivo* IL-4 production is independent of B7-dependent costimulation with high dose anti-CD3 antibody treatment but is dependent on costimulation following low dose anti-CD3 antibody treatment. Splenic IL-4 gene expression (top) was examined 90 minutes following treatment with various doses of anti-CD3 or hamster IgG antibody in the presence of CTLA4-Ig or the control fusion protein L6. Mice were given 100 μg of CTLA4-Ig or L6 24 h and 1 h prior to injection with anti-CD3 or hamster IgG antibody. The mice were euthanized and the spleens were removed 90 minutes after treatment. Total splenic RNA was isolated and examined for IL-4 mRNA expression by RT-PCR as stated in the legend to Figure 22. IL-4 protein was examined by the ELISPOT assay 4 h after treatment (bottom). Splenocytes were incubated on anti-IL-4 coated plates then removed and the plates incubated with a second biotinylated anti-IL-4 antibody followed by SA-alkaline phosphatase. The plates were covered with LMP agarose containing substrate and incubated overnight. The following day the number of spots were counted, each spot representative of an IL-4 producing cell. These data are expressed as the mean number of IL-4 producing cells per 10^6 splenocytes ± SEM and is representative of two experiments with three mice per group, * $p < 0.05$, ** $p < 0.001$.

Increased Splenic IL-4 Production is Blocked By CTLA4-Ig Following Low Dose But Not High Dose Anti-CD3 Antibody Treatment





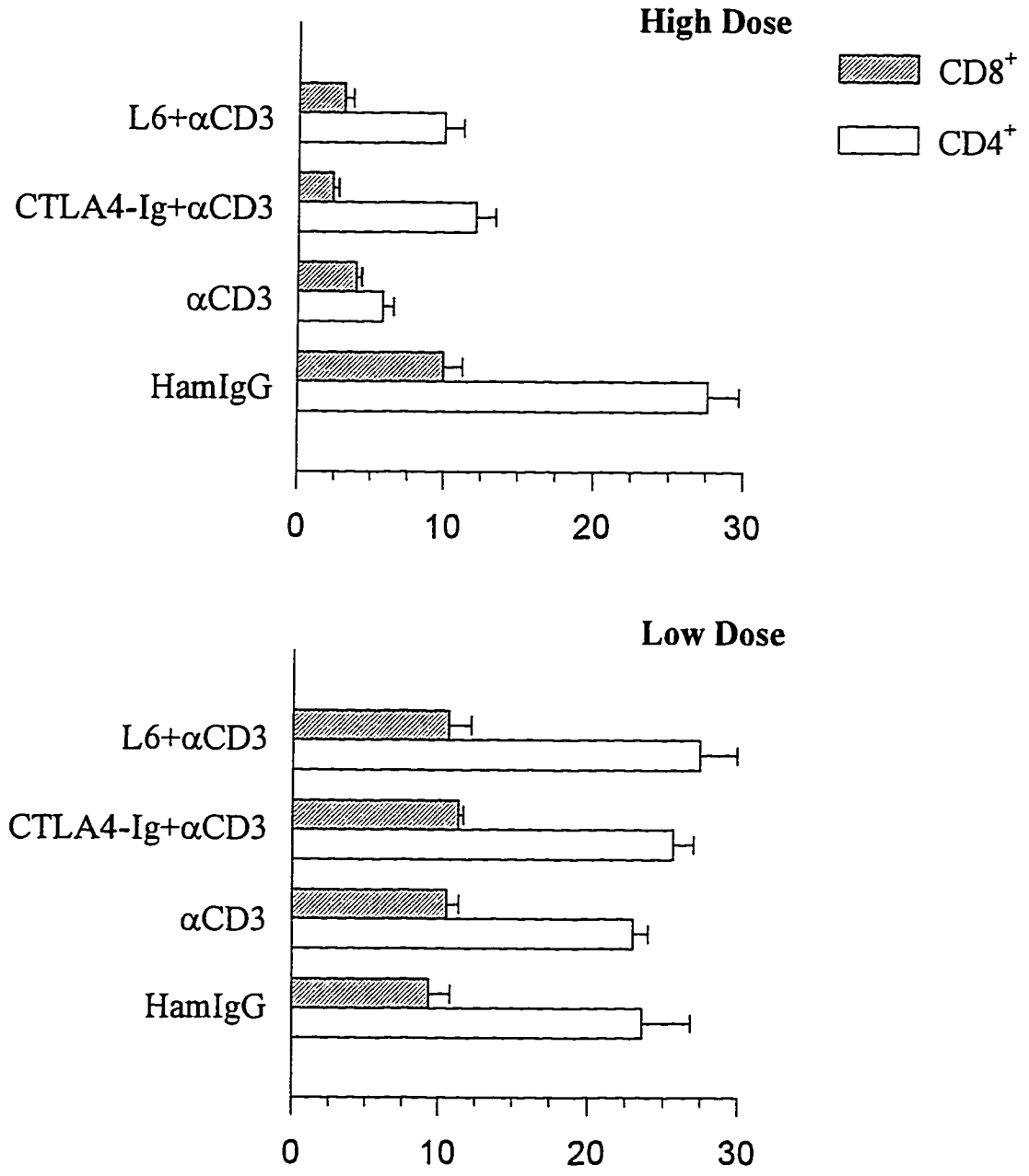
The role of endogenous B7-dependent T cell costimulation in preventing T cell depletion. Since B7-dependent costimulation is required for low-dose anti-CD3 antibody-induced expression of IL-4, the ability of CTLA4-Ig to prevent the loss of peripheral T cells was tested. BALB/c mice were injected with 200 μg CTLA4-Ig or L6 as previously described, together with 20 μg of hamster IgG or anti-CD3 antibody. T cell depletion was assessed in the spleen and lymph nodes four days after treatment by measuring the loss of peripheral T cells. CTLA4-Ig was unable to prevent depletion of peripheral $CD4^+$ or $CD8^+$ T cells by anti-CD3 antibody, compared to mice treated with the control fusion protein L6 (Figure 24 A). Comparison of T cell depletion between groups given anti-CD3 antibody alone or with CTLA4-Ig or L6 did not show statistically significant, ($p > 0.05$), differences. A similar experiment with a low-dose (0.1 μg) of anti-CD3 antibody did not produce statistically detectable, ($p > 0.05$), peripheral T cell depletion either in the presence of CTLA4-Ig or L6 (Figure 24 B). The inability of CTLA4-Ig to prevent high-dose anti-CD3 antibody-induced T cell depletion was puzzling, since a primary function of B7-dependent costimulation is thought to be the induction and maintenance of IL-2 expression. Since it was previously demonstrated that the IL-2 pathway was required for $CD8^+$ T cell apoptosis (Figure 21), this result suggests that high-dose anti-CD3 antibody-stimulation alone is able to induce sufficient IL-2 production to delete $CD8^+$ T cells.

Since measuring the depletion of T cells is not equivalent to measuring apoptosis, a similar experiment was conducted using the FLANUL assay to measure increases in the appearance of apoptotic $Thy1^+$, $B220^+$, and $Thy1^+$ $B220^+$ cells two days following treatment

with low and high-doses of anti-CD3 antibody in the presence and absence of CTLA4-Ig (Figure 25 and Table 7). These data show a similar increase in apoptotic $Thy1^-$, $B220^-$, and $Thy1^- B220^-$ cells both in the presence and the absence of CTLA4-Ig following high-dose anti-CD3 antibody-treatment. The low-dose of anti-CD3 antibody was unable to induce a statistically significant, ($p > 0.05$), increase in apoptotic cells over control antibody-treated mice. The results of these experiments support the T cell depletion data because CTLA4-Ig was unable to prevent the induction of apoptosis. Also, apoptosis could not be detected at the (0.1 μg), low-dose, of anti-CD3 antibody treatment where the expression of IL-4 becomes dependent on B7-dependent costimulation. These experiments suggest that apoptosis, as well as induction of cytokine production occurs independently of the costimulatory pathway when a strong TCR-signal is provided to the T cell.

Figure 24. CTLA4-Ig cannot block anti-CD3 antibody-induced peripheral T cell depletion. BALB/c mice were injected with 200 μg of CTLA4-Ig or control protein L6 in two-100 μg doses. The first dose was given 24 h before and the second dose concurrently with 20 μg hamster IgG or anti-CD3 antibody (A) or 0.1 μg (B). The mice were euthanized 4 days later and splenocytes were labeled with antibodies for the T cell markers CD4 and CD8. The number of $CD4^+$ or $CD8^+$ cells was calculated, using a live gate, by multiplying the percent of $CD4^+$ or $CD8^+$ cells by the total number of recovered splenocytes. The mean (n=3) ± SEM for the number of recovered $CD4^+$ or $CD8^+$ positive lymphocytes is displayed, these data are representative of two independent experiments.

The Deletion of Peripheral T cells by Anti-CD3 Antibody Does Not Require B7-Dependent Costimulation



Number of Recovered Cells x 10^6

Figure 25. Anti-CD3 antibody-induced apoptosis is not blocked by CTLA4-Ig. BALB/c mice were given 100 μg of CTLA4-Ig or L6 control fusion protein 24 h before and another 100 μg of CTLA4-Ig or L6 concurrently with 20 μg or 0.1 μg of anti-CD3 antibody or hamster IgG. Forty-eight h later the animals were euthanized and spleen cells were prepared and surface labeled with anti-Thy1-PE, and anti-B220-FITC. Free 3' DNA ends were labeled with biotinylated dUTP and streptavidin-APC as described in the Materials and Methods. The cells were analyzed on a Coulter EPICS Elite analyzer and the histograms represent: (A) 20 μg hamster IgG, (B) 20 μg anti-CD3 antibody, (C) 0.1 μg anti-CD3 antibody, in the absence (left side) or presence (right side) of CTLA4-Ig. The histograms are representative of one of three mice per group. Numbers represent the percent of labeled splenocytes in that quadrant. Data from a similar experiment are displayed in Table 7.

CTLA4-Ig Does Not Prevent Anti-CD3 Antibody-Induced Apoptosis

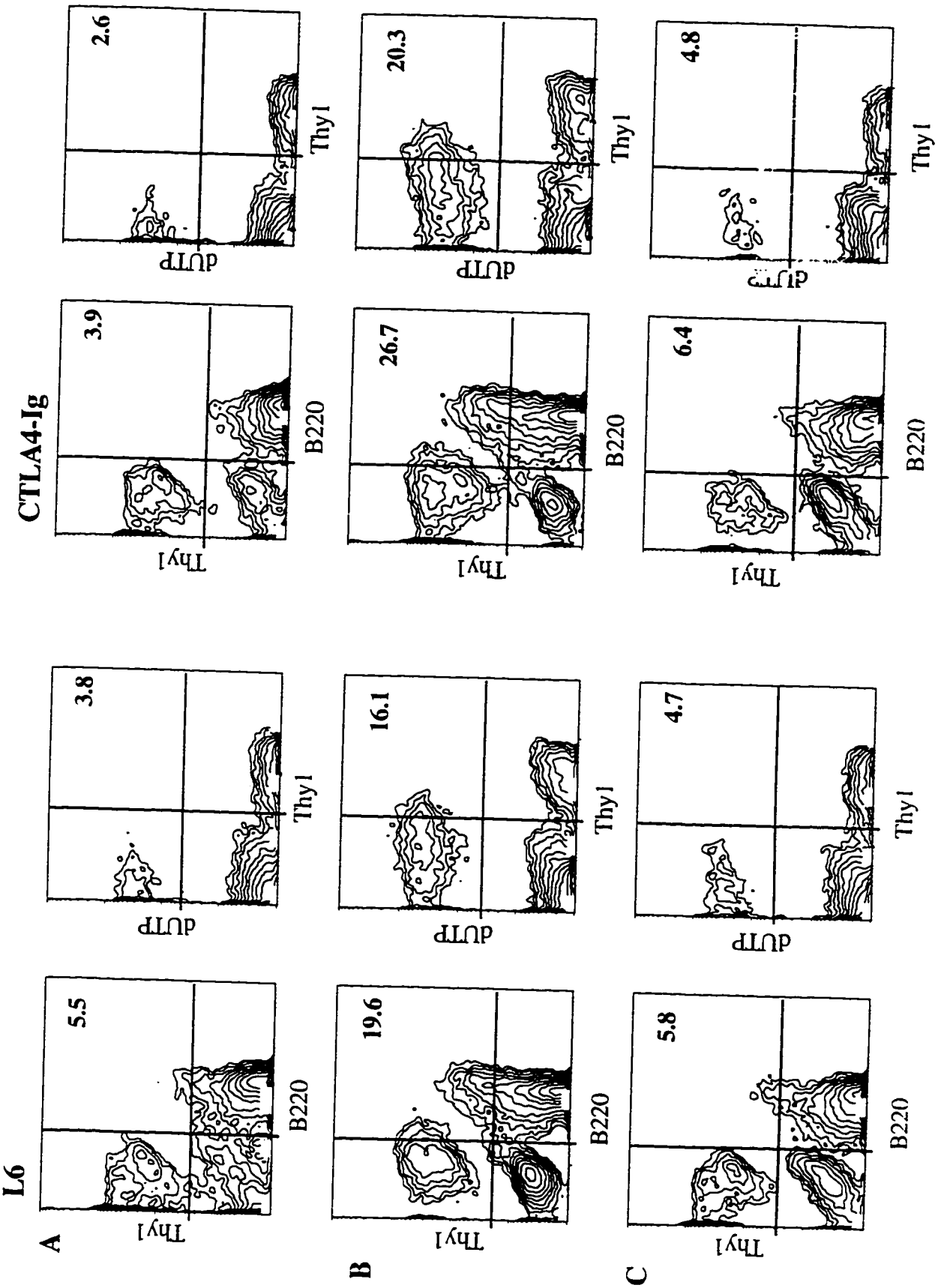


Table 7. Cell Surface Phenotype of Splenic Lymphocytes Following Treatment With Anti-CD3 Antibody and CTLA4-Ig.

Mean ± SEM % Lymphocyte Staining[a]						
	L6			**CTLA4-Ig**		
Group[b] Treatment	%Thy1$^-$ B220$^+$ UTP$^+$	%Thy1$^-$ B220$^-$ UTP$^+$	%Thy1$^-$ B220$^+$ UTP$^+$	%Thy1$^-$ B220$^+$ UTP$^+$	%Thy1$^-$ B220$^-$ UTP$^-$	%Thy1$^-$ B220$^-$ UTP$^-$
hamIgG	9.4 ± 0.5	0.6 ± 0.1	3.9 ± 0.7	8.8 ± 0.3	0.5 ± 0.1	5.3 ± 0.8
anti-CD3 (high dose)	9.4 ± 0.7	2.2 ± 0.2	12.8 ± 1.6	9.4 ± 0.7	2.9 ± 0.1	12.1 ± 0.9
anti-CD3 (low dose)	7.5 ± 0.5	0.8 ± 0.1	3.2 ± 0.5	7.1 ± 0.6	0.8 ± 0.1	3.8 ± 0.3

[a] Splenic cell preparations were stained with anti-B220-FITC and anti-Thy1-PE then free 3' DNA ends were labeled with biotinylated dUTP followed by SA-APC. Gates were established to exclude monocytes, RBCs, cell doublets and debris and to include lymphocytes and lymphocytes with reduced FSC. Mean and SEM for BALB/c mice, four per group, are displayed for each treatment.

[b] Mice were given iv injections of 100 μg of CTLA4-Ig or L6 24 h and again 1 h before treatment with 20 μg hamster IgG or anti-CD3 antibody and 48 h later the mice were euthanized.

Activation of antigen presenting cells with LPS fails to prevent anti-CD3 antibody-induced apoptosis of T cells. LPS has pleiotropic effects *in vivo* including the induction of APC cytokine production and costimulatory ligand expression including B7-1 (CD80) and B7-2 (CD86) (Chen and Nabavi.1994). LPS rescues T cells from superantigen-induced T cell apoptosis in a process which requires APC-dependent production of TNF-α (Vella *et al.*,1995). These results suggest that LPS may be able to block or divert activation-induced apoptosis through the up-regulation of endogenous costimulatory ligands and/or the production of cytokines by APCs. Therefore, two types of experiments using LPS to up-regulate APC costimulatory and/or accessory signaling which, in turn, may rescue T cells from anti-CD3 antibody-induced deletion were performed. The first approach was to pre-activate APCs with LPS to increase the expression of endogenous costimulatory molecules and cytokines prior to administration of anti-CD3 antibody. The second approach was to provide costimulatory or accessory signals by activating antigen presenting cells/cytokine production after treatment with anti-CD3 antibody.

The first approach entailed injecting BALB/c mice with various doses of LPS 24 h, 6 h or in conjunction with 20 μg of PBS or anti-CD3 antibody and four days later measuring T cell depletion by counting the remaining $CD4^+$ and $CD8^+$ splenic and lymph node cells. Administration of 1-25 μg of LPS failed to have a significant effect on the loss of splenic $CD4^+$ or $CD8^+$ T cells when given with or 6 h prior to anti-CD3 antibody. When 12.5 μg or 6 μg of LPS was given 24 h prior to anti-CD3 antibody-treatment there was a statistically significant, ($p < 0.05$), effect (Figure 26). These data suggest that LPS given prior to anti-

CD3 antibody treatment provides marginal protection against T cell depletion in a dose and time restricted manner. These results were observed in two of three experiments.

The subsequent administration of LPS 6 h or 18 h after treatment with anti-CD3 antibody failed to rescue peripheral T cells from anti-CD3 antibody-induced deletion. BALB/c mice were injected with 0.25, 1.0, 4 or 12 μg of LPS, and four days later peripheral T cell depletion was measured by counting $CD4^+$ and $CD8^+$ splenic and lymph node T cells (Figure 27). These data show that LPS given after anti-CD3 antibody, fails to maintain peripheral T cell numbers. In fact, any dose of LPS greater than 4 μg, when given more than 6 h after anti-CD3 antibody-treatment, was lethal (data not shown). Mice given 25 μg of LPS alone did not die. The LPS used in both experiments was biologically active *in vivo* as demonstrated by increased cell size and MHC class II expression on $B220^+$ cells 18-36 h following LPS-treatment (Figure 28). Together, these data suggest that LPS is either unable to provide the necessary costimulatory or accessory signaling to prevent anti-CD3 antibody-induced T cell deletion or that the inflammatory effects of LPS, in conjunction with anti-CD3 antibody, are so severe that they lead to the death of the animal. This finding is similar to the observation that exogenous IL-2 complexes also sensitized mice to anti-CD3 antibody-induced death.

High doses of LPS (25 μg) were unable to prevent anti-CD3 antibody-induced T cell depletion in contrast to the observations in superantigen-treated mice (Vella *et al.*, 1995). One possible explanation could be the difference in mice used in the experiments. Since BALB/c are hyper-responsive to LPS, the failure of T cells to be rescued from anti-CD3 antibody-induced depletion could be due to LPS-mediated toxicity. Therefore, the

administration of LPS after treatment with anti-CD3 antibody in hypo-responsive C3H/OuJ mice was examined. Mice were given 1, 4 or 12 μg of LPS 12 h following anti-CD3 antibody-treatment and four days later surviving peripheral $CD4^+$ and $CD8^+$ cells were counted. The results of this experiment were essentially the same as in mice with the BALB/c background. Anti-CD3 antibody treated mice, receiving 12 μg of LPS, died prior to the completion of the experiment and T cell depletion was not prevented at any dose of LPS. The failure to rescue T cells with LPS, in both BALB/c and C3H/OuJ mice, suggests that intrinsic differences in the LPS response are not important in preventing anti-CD3 antibody-induced deletion .

Figure 26. Pretreatment with LPS shows a differential ability to rescue peripheral T cells from anti-CD3 antibody-induced T cell depletion. BALB/c mice were given iv injections of 25, 12.5, 6 or 1 μg LPS 24 h prior to administration of 20 μg of anti-CD3 antibody or 25 μg of LPS alone. Four days after treatment with anti-CD3 antibody the mice were euthanized and cell suspensions prepared from the spleens. The splenocytes were prepared and stained with antibodies to CD4 or CD8 as described in the legend to Figure 1. These data represent the mean (n=3) ± SEM for the recovered cells in each treatment group. This experiment is representative of three similar experiments, * $p < 0.05$.

Pretreatment with LPS Does Not Prevent Deletion of Splenic T cells by Anti-CD3 Antibody

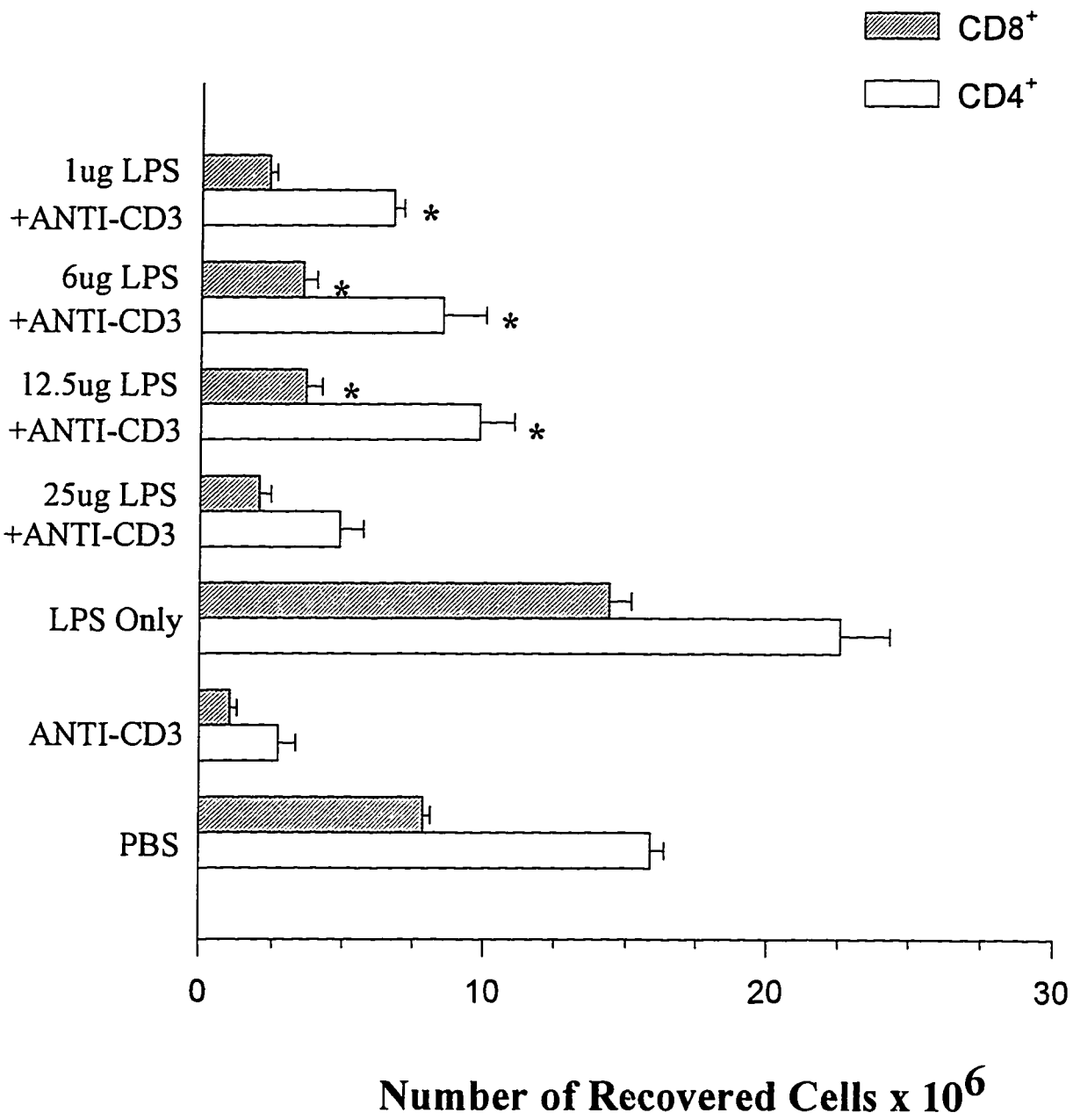


Figure 27. Administration of LPS after anti-CD3 antibody-treatment does not block peripheral T cell depletion. BALB/c mice were given iv injections of 0.25, 1.0, 4 or 12 μg LPS 6 h or 18 h after administration of 20 μg of anti-CD3 antibody or 12 μg of LPS alone. Four days after treatment with anti-CD3 antibody the mice were euthanized and cell suspensions prepared from the spleen. Mice given 4 or 12 μg of LPS after anti-CD3 antibody-treatment at both time points died prior to the completion of the experiment. The splenocytes of the surviving mice were prepared, labeled and counted as described in the legend to Figure 26 These data represent the mean (n=3) ± SEM for the number of recovered $CD4^+$ and $CD8^+$ cells in each treatment group. This experiment is representative of two similar experiments.

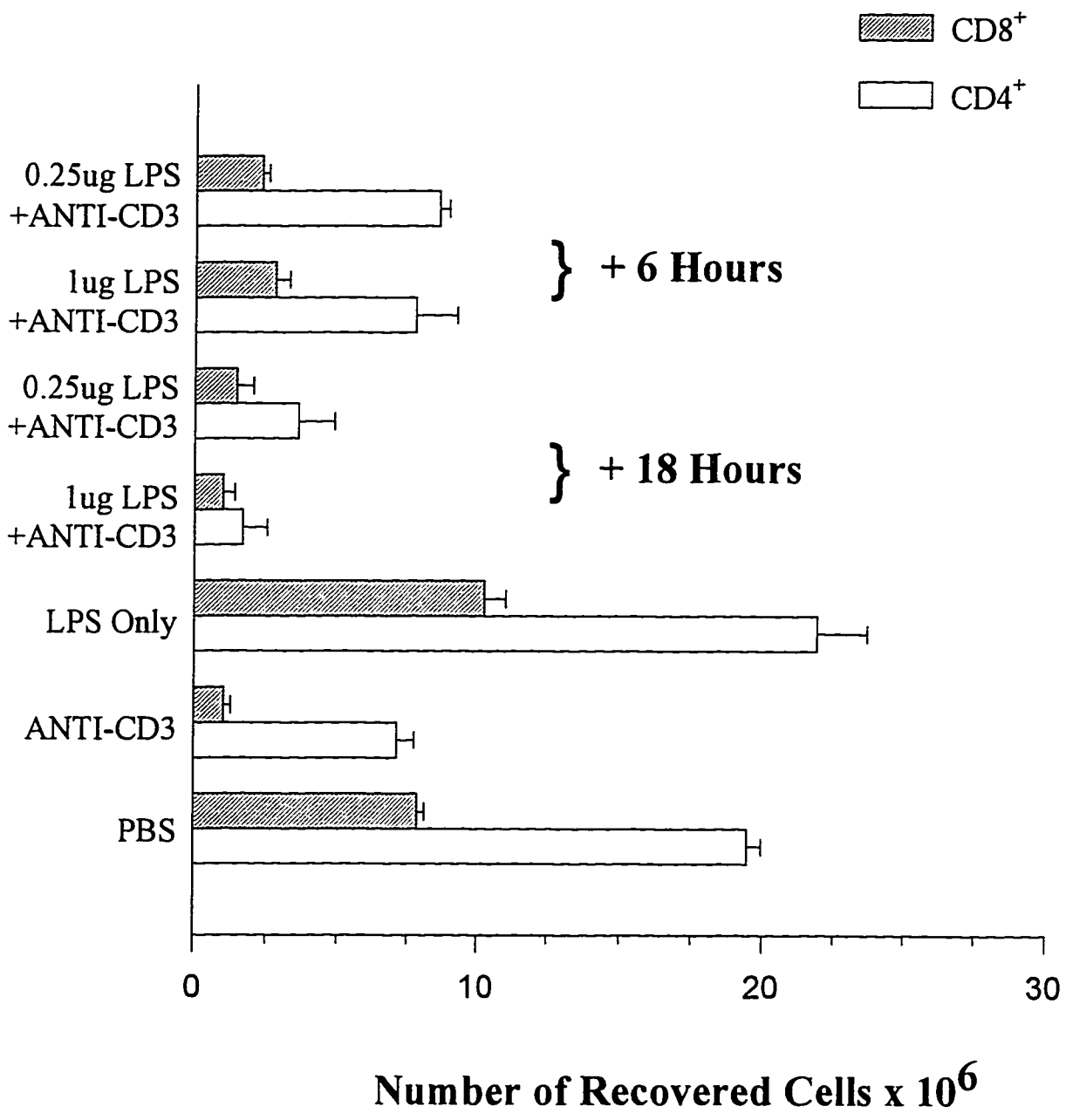
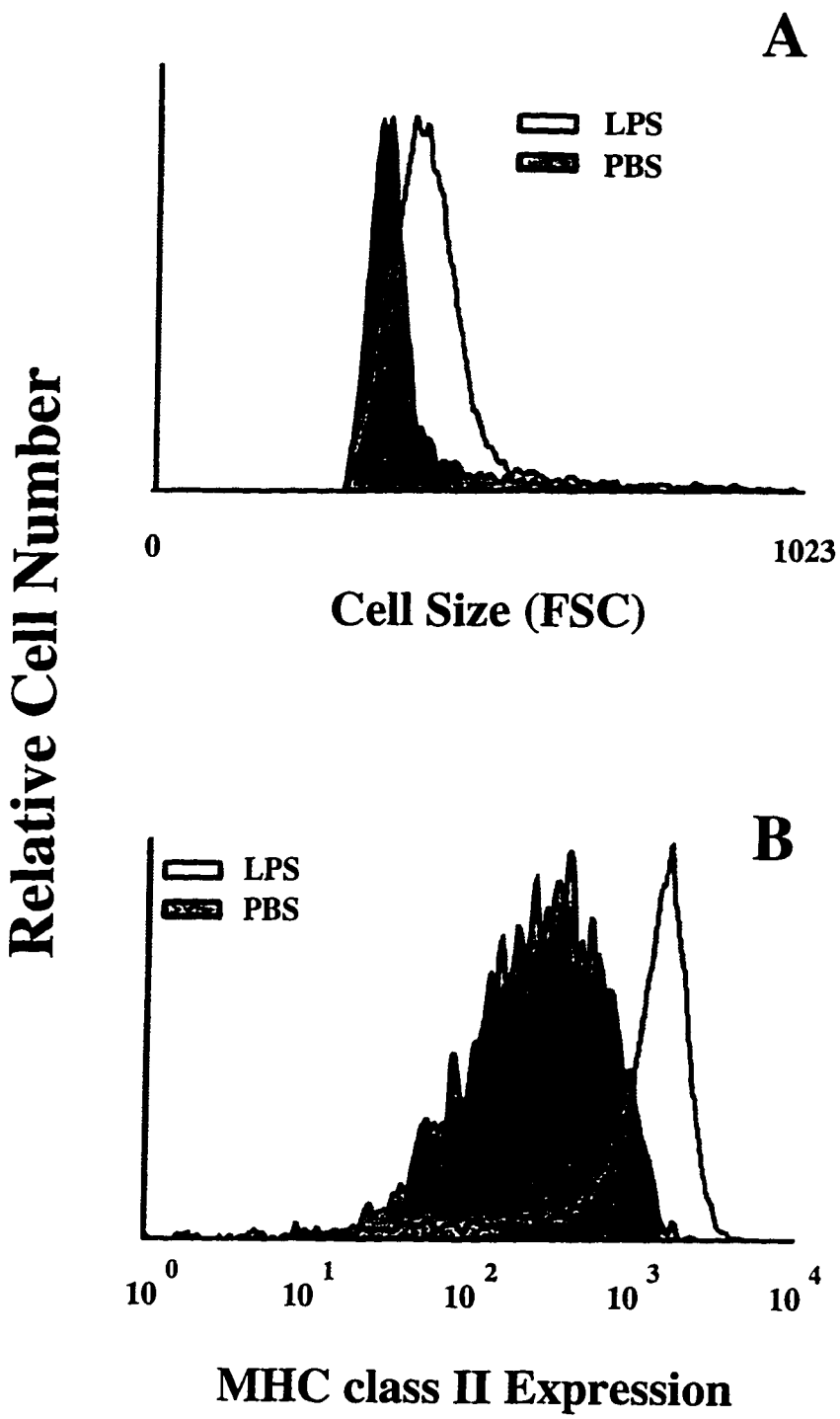

LPS Does Not Rescue T cells
From Anti-CD3 Antibody-Induced Deletion
CD8+
CD4+
0.25ug LPS +ANTI-CD3
1ug LPS +ANTI-CD3
0.25ug LPS +ANTI-CD3
1ug LPS +ANTI-CD3
LPS Only
ANTI-CD3
PBS
+ 6 Hours
+ 18 Hours
0
10
20
30
Number of Recovered Cells x 10^6

Figure 28. LPS activates B cells. BALB/c mice were given 25 μg of *E. coli* K235 LPS or sterile PBS and euthanized 24 h later. Splenocytes were prepared as described in the legend to Figure 26 and labeled with antibodies to B220 and MHC class II. The cells were analyzed on a Coulter EPICS Elite to measure increases in FSC and MHC class II expression of $B220^-$ gated cells. A) Representative linear-scale histogram of FSC of $B220^-$ PBS-treated splenocytes (gray) *vs.* LPS treated $B220^-$ splenocytes (open). B) Representative log-scale histogram of MHC class II fluorescence from $B220^-$ PBS-treated splenocytes (gray) *vs.* LPS treated $B220^-$ splenocytes (open). These data are representative of two experiments.

LPS Treatment Activates B Cells



Discussion.

Summary. In this study, the fate of T cells stimulated *in vivo* with anti-CD3 antibodies, a treatment known to induce T cell depletion, (Hirsch *et al.*,1988; Hirsch *et al.*,1989) was examined. Activation of T cells *in vivo* with anti-CD3 antibody has been hypothesized to mimic antigen specific activation of T cells leading to AICD (Kabelitz *et al.*,1993; Russell *et al.*,1991). My data support this hypothesis by demonstrating that peripheral T cells stimulated through their TCR by high doses of anti-CD3 antibody undergo AICD *in vivo* . Findings from this thesis showed that following *in vivo* treatment with anti-CD3 antibody, peripheral T cells express a large number of cytokines including IL-2, IL-4, IFN-γ, and TNF-α but not IL-10; they also express increased levels of activation markers including IL-2R, *fas* and *fasL* and initially have increased cell size. These cells subsequently displayed the characteristics of apoptosis including condensation of the nucleus, chromatin compaction, reduction in cell volume, and DNA fragmentation. Associated with this pathway was the detection of an unusual peripheral T cell phenotype characterized by the intermediate expression of the T cell marker Thy1, expression of the B cell marker B220 and loss of the T cell markers CD4 and CD8. This phenotype is similar to the phenotype that accumulates in *lpr* and *gld* mice (Watanabe-Fukunaga *et al.*, 1992) and thus may represent a pre-apoptotic surface phenotype for T cells.

A role for the B7-dependent costimulatory pathway in anti-CD3 antibody-induced cytokine production, but not apoptosis, was demonstrated. The addition of the fusion protein CTLA4-Ig was shown to block IL-2 and IL-4 gene expression at low doses, but not high

doses, of anti-CD3 antibody stimulation. Additionally, CTLA4-Ig did not alter the high dose anti-CD3 antibody-induced apoptosis, suggesting a role for endogenous costimulation in dose-dependent anti-CD3 antibody-induced cytokine production but not in apoptosis. Blocking the IL-2 pathway *in vivo* prevented anti-CD3 antibody-induced $CD8^+$ but not $CD4^+$ T cell AICD, which suggests that the induction of IL-2 is required for anti-CD3 antibody-induced deletion of $CD8^+$ T cells. Endogenous up regulation of costimulatory signals, including cytokine production and up regulation of B7 molecules, to rescue T cells from the AICD pathway was attempted by treatment of mice with LPS. Several experiments in which the dose or timing of LPS administration were altered failed to divert high dose anti-CD3 antibody-induced apoptosis suggesting that high dose TCR signaling may be irreversibly commit T cells to the AICD pathway. Cytokine production could be detected at both the low and the high dose of anti-CD3 antibody treatment while apoptosis could only be detected at the high dose. This suggests that the quantitative level of the signal delivered through the TCR must be critical to initiate T cell activation leading to AICD.

Collectively, my data support a model of immune regulation in which AICD is initiated in T cells which are inappropriately stimulated by a strong signal through the TCR. This pathway does not require CD28-dependent signals but interestingly, $CD8^+$ but not $CD4^+$ cells require an IL-2-dependent signal to initiate AICD. In addition, the apoptotic T cells have a cell surface phenotype similar to aberrant lymphocytes which accumulate in mice with genetic mutations in apoptosis associated genes. The implications of these findings will be discussed below.

Anti-CD3 antibody-mediated T cell activation leading to cytokine production or apoptosis at a low or high dose. T cell activation with anti-CD3 antibody is frequently used as a model for cognate TCR signaling in both *in vitro* and *in vivo* studies (Weiss.1993). Previous studies have revealed several important aspects of TCR signaling including the importance of APCs (Groux *et al.*,1993; Parren *et al.*,1992; Wu *et al.*,1995; Ceuppens *et al.*,1985), cross-linking of the TCR (Hughes *et al.*,1994; Henrickson *et al.*,1994; Herold *et al.*,1992; Hirsch *et al.*,1990; Hirsch *et al.*,1991; Anasetti *et al.*,1992; Alegre *et al.*,1994; Woodle *et al.*,1991), costimulation by nonantigen-specific cell-surface molecules (Bluestone *et al.*,1993), cytokine signaling (Lenardo.1991; Groux *et al.*,1993; Yanagida *et al.*,1994; Alegre *et al.*,1990), as well as the activation state, and the maturity of the responding T cell (Smith *et al.*,1989; Shi *et al.*,1991; Schwinzer *et al.*,1992; Groux *et al.*,1993; Wesselborg *et al.*,1993; Lightstone *et al.*,1993). *In vitro* studies demonstrated that plate bound anti-CD3 antibody signaling of activated T cells or hybridomas leads to apoptosis. In contrast, stimulation of freshly isolated naive-resting T cells by immobilized anti-CD3 antibody induces anergy (Wesselborg *et al.*,1993). *In vivo* anti-CD3 antibody treatment causes suppression of T cell mediated allograft rejection through depletion of T cells from peripheral lymphoid tissue and both $CD4^+$ and $CD8^+$ TCR-$\alpha\beta$ and TCR-$\gamma\delta$ cells are deleted by this treatment (Hirsch *et al.*,1988; Cron *et al.*,1989; Janssen *et al.*,1991). Collectively these studies suggest that anti-CD3 antibody stimulation may cause T cell AICD *in vivo*.

My findings support the hypothesis that AICD is the mechanism of T cell depletion observed following *in vivo* administration of anti-CD3 antibody. T cell depletion occurred

in a dose-dependent manner, four days following treatment with anti-CD3 antibody (Figures 1, 2). Consistent with the hypothesis that T cell apoptosis is an activation-dependent event (Cohen *et al.*, 1992), it was found that deletion of T cells from the spleen and lymph nodes was preceded by activation of a large percentage of these cells during the first 24 h after treatment with anti-CD3 antibody as detected by increased cell size and surface IL-2R expression (Figure 3). This observation is consistent with previous data which demonstrated that *in vivo* treatment with anti-CD3 antibody induced an increase in IL-2R expression on peripheral T cells following administration of large doses (400 μg) of anti-CD3 antibody (Hirsch *et al.*, 1989).

Previous *in vitro* studies demonstrated that anti-CD3 antibody treatment induces apoptosis of Th1 and Th2 clones and T cell lines (Williams *et al.*, 1992; Luqman and Bottomly.1992; Schwartz.1990; Radvanyi *et al.*, 1993; Burstein *et al.*, 1992). *In vivo* studies demonstrated that thymocytes undergo anti-CD3 antibody-induced apoptosis (Shi *et al.*, 1991; Groux *et al.*, 1993), and my studies have confirmed these results (Figures 4, 5). To date, no direct evidence has been provided to demonstrate that anti-CD3 antibody-induced apoptosis occurs in peripheral T cells. My results provide evidence to support an apoptotic mechanism of cell death of peripheral T cells following anti-CD3 antibody treatment. This evidence came from three assays which required a short term *in vitro* incubation. The first assay demonstrated apoptotic morphology of individual T cells by fluorescent microscopy. The apoptotic morphology included shrinkage of the nucleus and nuclear chromatin compaction (Figure 6 and Table 3). The second assay was used to detect DNA fragmentation, an early marker of apoptosis, that results from cleavage of the cell's DNA

into 200 bp oligonucleosomal fragments. Following treatment with anti-CD3 antibody, $Thy1^+$ splenocytes from anti-CD3 antibody treated but not control treated mice displayed the characteristic ladder of fragmented DNA seen in apoptotic cell populations (Figure 7). The third assay used to detect apoptosis is a novel modification of the TUNEL assay (Gavrieli *et al.*, 1992). This new FLANUL (Fluorescent Labeling and Nick translation UTP Labeling) assay combines the *in situ* nick translation labeling of free 3' ends of DNA with fluorescent labeling of cell surface markers allowing the detection and immunotyping of individual apoptotic cells. This assay confirmed previous observations that cells with hypodiploid DNA content (as detected with propidium iodide) also had reduced FSC (Darzynkiewicz *et al.*, 1992) and demonstrated that cells with fragmented DNA, *i.e.*, apoptotic cells, are almost exclusively found in a population with reduced FSC following treatment with anti-CD3 antibody (Figure 8). This technique may be useful for future *in vivo* studies in which the fate of specific T cell subsets is studied.

The results of this study demonstrate apoptosis of T cells following *in vivo* treatment with anti-CD3 antibody but does not exclude the possibility of T cell depletion due to emigration. Significant T cell depletion could be detected four days after anti-CD3 antibody treatment and all three apoptosis assays could detect apoptosis from one to four days after treatment. The optimal time to assay for apoptosis was 48 h post treatment. However, any time between 24-72 h after anti-CD3 antibody administration allowed the detection of significant levels of apoptosis, suggesting that apoptosis in this model occurs at low levels over an extended period. Anti-CD3 antibody-induced T cell activation is a good model to study T cell deletion since anti-CD3 antibody binds to most T cells and thus initiates a

similar response in a large pool of responder cells. It has been hypothesized that the T cell deletion following anti-CD3 antibody treatment may be similar to the fate of T cells activated by APC in the absence of costimulatory and accessory signals required for the proper development of T effector cells (D'Adamio *et al.*, 1993; Wu *et al.*, 1995). My results confirm this hypothesis. The inability of previous investigators to detect apoptosis of peripheral T cells following anti-CD3 antibody or superantigen treatment *in vivo* is most likely a result of insensitive assays, or in the case of superantigens, an inability to detect the small apoptotic T cell population over an extended time course (Alegre *et al.*, 1991; Hirsch *et al.*, 1988; Kawabe and Ochi.1991; MacDonald *et al.*, 1991). My novel modification of the TUNEL assay to include identification of the cell surface phenotype of apoptotic cells increases the utility of this technique for use in numerous *in vivo* models when the fate of specific cell populations must be determined. This technique is more sensitive than other assays used to detect apoptosis; it is specific in that apoptotic cell death can be discriminated from necrotic cell death, and it allows the direct quantitation of cell populations undergoing AICD.

T cell cytokines may affect the outcome of T cell signaling leading to either the development of effector cell functions or AICD. Cytokines are potent peptide messengers with known roles in T cell proliferation and accessory signaling (Oppenheim *et al.*, 1991; Arai *et al.*, 1990). Cytokine gene expression *in vivo* has been observed prior to the induction of T cell depletion with mitogenic forms of anti-CD3 antibody (Scott *et al.*, 1990; Hirsch *et al.*, 1989; Flamand *et al.*, 1990; Alegre *et al.*, 1991; Ferren *et al.*, 1990;). Nonmitogenic forms of anti-CD3 antibody do not induce cytokine expression and induce tolerance through a

nondeletional mechanism (Hughes *et al.*,1994; Hirsch *et al.*,1991; Alegre *et al.*,1994; Woodle *et al.*,1991). It was also demonstrated that the elevations in cytokines following mitogenic anti-CD3 antibody treatment were not due to LPS contamination because the results were reproducible in LPS resistant C3H/HeJ mice but not in LPS sensitive nude mice (Ferren *et al.*,1990). My data confirmed and extended these results by demonstrating a rapid increase in expression of IL-2, IL-4, TNF-α and IFN-γ but not IL-10 in both the spleen and lymph nodes of BALB/c mice following treatment with 20 μg of anti-CD3 antibody (Figures 17 and 18). Therefore, a role for certain cytokines including TNF-α, IFN-γ, and the T cell growth factors, IL-2 and IL-4, in anti-CD3 antibody-induced apoptosis can be hypothesized.

IFN-γ has been implicated in the prevention of apoptosis in Th1 cells (Liu and Janeway, Jr.1990) but others have not found data to support this model (Russell *et al.*,1992). Conversely, IFN-γ has been implicated in inducing apoptosis of peripheral human T cells as anti-IFN-γ antibodies were shown to prevent anti-CD3 antibody-induced apoptosis *in vitro* (Groux *et al.*,1993). It was also reported that IFN-γ is required for anti-CD3 antibody-induced apoptosis of Th1 cells (Liu and Janeway, Jr.1990), but no other reports have expanded this finding. Anti-CD3 antibody-induced IFN-γ expression *in vivo* may occur in a cascade manner, requiring TNF-α, as anti-TNF-α antibody can prevent the expression of IFN-γ (Ferran *et al.*,1991). Since nonmitogenic anti-CD3 antibody does not induce IFN-γ expression or T cell depletion but does induce T cell anergy, the IFN-γ may be important in initiating apoptosis. Interestingly, my data show that IFN-γ mRNA expression remained elevated for four days after anti-CD3 antibody treatment. Intervention

experiments were not attempted; therefore a definitive role for IFN-γ in anti-CD3 antibody-induced apoptosis remains to be determined.

The induction of IL-4 by *in vivo* treatment with anti-CD3 antibody was noted to occur in a specialized $CD4^-$ $NK1.1^+$ and $CD3^{low}$ T cell subset. These cells produce a large number of cytokines in the bone marrow and thymus and are hypothesized to play a regulatory role in the adaptive immune response (Arase *et al.*, 1993). Recent findings suggest that this T cell subset produces all the IL-4 induced by low dose *in vivo* anti-CD3 antibody treatment. This rapid IL-4 production further suggests that this subset may serve a regulatory function in the initiation of a Th2-like immune response (Yoshimoto and Paul.1994). My findings agree with previous observations in that these cells were found to produce the majority of IL-4 mRNA following treatment with a low dose of anti-CD3 antibody. However, by using the more sensitive RT-PCR technique it was found that $CD4^-$ $NK1.1^-$ cells also produce IL-4 mRNA, in contrast to the previous findings. The IL-4 mRNA production by $CD4^-$ $NK1.1^-$ cells is more significant at higher doses of anti-CD3 antibody and becomes independent of the costimulatory pathway (Figures 20, 22, 23). The cytokine production by these cells could be significant because the $CD4^-$ $NK1.1^-$ cells become susceptible to anti-CD3 antibody-induced apoptosis during increased IL-4 expression. Since $CD4^-$ $NK1.1^-$ cells express lower levels of CD3, these data also suggest that the difference in IL-4 produced by $CD4^+$ $NK1.1^-$ cells and $CD4^+$ $NK1.1^+$ cells is due to qualitative differences in anti-CD3 antibody-mediated signaling which becomes less significant at higher levels of TCR cross-linking. The results also argue against the hypothesis that IL-4 has a role in the prevention of anti-CD3 antibody-induced apoptosis, since the majority of T cells undergo apoptosis in

the face of substantial IL-4 production (Figure 17-19 and Table 6). The addition of exogenous IL-4 complexes also failed to prevent anti-CD3 antibody-induced T cell depletion again suggesting that IL-4 is unable to prevent AICD. But, like IL-2 production, the timing of the IL-4 production could have a critical effect on the outcome of anti-CD3 antibody-induced apoptosis. Specifically, IL-4 production occurs early after anti-CD3 antibody treatment then rapidly declines followed by apoptosis of the majority of T cells. If IL-4 was provided later in the immune response, it may be possible to rescue T cells from apoptosis.

IL-2 has also been implicated in the prevention of apoptosis, and in priming T cells for apoptosis (Mueller *et al.*, 1989; Lenardo.1991; Boehme and Lenardo.1993). IL-2 is an important T cell growth factor and is required *in vitro* to prevent anti-CD3 antibody-induced T cell anergy, but the timing of IL-2 availability is also critical to proliferation or apoptosis. Previous studies have shown that the timing of growth factor administration is critical since *in vitro* pretreatment with the T cell growth factors IL-2 and IL-4 sensitized the A.E7 T cell clone to anti-CD3 antibody-induced apoptosis. The mechanism for sensitizing T cells to apoptosis was IL-2 or IL-4-induced entry into the cell-cycle. Activated cells underwent apoptosis while in S phase of the cell-cycle in a process termed "propriocidal regulation" (Boehme and Lenardo.1993). T cells stimulated *in vitro* by anti-CD3 antibody alone produce IL-2 for a brief period then subsequently become anergic and fail to produce IL-2. Activated T cells, previously exposed to IL-2, undergo AICD when restimulated with anti-CD3 antibody or superantigens (Kabelitz *et al.*, 1993; Russell *et al.*, 1991; Boehme and Lenardo.1993). Based on these findings, and my data demonstrating that anti-CD3 antibody-induced early expression of IL-2 mRNA in T cells (Figure 19), it was hypothesized that

early, unsustained IL-2 exposure may sensitize T cells to AICD. The administration of IL-2/anti-IL-2 complexes, with or after high-dose anti-CD3 antibody administration, was lethal to mice. At lower doses these IL-2 complexes had no effect on peripheral T cell deletion suggesting that the sustained presence of IL-2 is unable to rescue T cells from apoptosis. My finding that pretreatment with anti-IL-2/anti-IL-2R antibodies inhibited $CD8^+$ but not $CD4^+$ T cell AICD following anti-CD3 antibody injection suggests that IL-2 is differentially required for activation leading to apoptosis in these two cell populations (Figure 21). A primary function of $CD8^+$ T cells is cytotoxic destruction of cells displaying allogeneic MHC class I or MHC class I with a foreign peptide epitope. Therefore, pathogens could exploit the immune system by inducing nonspecific activation of $CD8^+$ T cells encountering an infected target cell without proper costimulation or T cell help by inducing the expression of IL-2. Prevention of $CD8^+$ T cell activation by IL-2-dependent AICD may be a mechanism to prevent autoimmunity or immune deviation. Apoptosis of $CD4^+$ T cells independently of IL-2 may be due to the importance of these cells in initiation of the cell-mediated immune response and thus reflect the increased importance in regulating these cells through a lower AICD initiation threshold. It would probable be advantageous to delete a portion of normal $CD4^+$ T cells by AICD rather then increasing the risk of autoreactive T cell activation. This hypothesis is consistent with the idea that activation of naive $CD4^+$ T cell requires cognate antigen signaling through the TCR, CD28 dependent costimulation and the sustained presence of cytokine growth factors such as IL-2. Since costimulation is important for T cell activation, a role for B7-dependent costimulation in AICD was hypothesized.

The role of T cell costimulation in AICD and apoptosis. The initial evidence suggesting that costimulation may play a role in the response of T cells to murine anti-human anti-CD3 antibody was shown through several *in vitro* studies in which surface cross-linking by a FcγR-bearing APC was required (Parren *et al.*, 1992; Anasetti *et al.*, 1992; Alegre *et al.*, 1994; Woodle *et al.*, 1991; Wu *et al.*, 1995; Ceuppens *et al.*, 1985; Woodle *et al.*, 1991). These studies are supported by parallel studies in mice with a hamster anti-mouse anti-CD3 antibody with homologous functional activity (Leo *et al.*, 1987; Herold *et al.*, 1992; Hirsch *et al.*, 1990; Hirsch *et al.*, 1991; Hughes *et al.*, 1994; Henrickson *et al.*, 1994; Hirsch *et al.*, 1991). In mice, the use of mitogenic 145-2C11 anti-CD3 antibody induces T cell activation, cytokine production, deletion of T cells, and suppression of the rejection of skin grafts (Hirsch *et al.*, 1988; Hirsch *et al.*, 1989). The use of $F(ab')_2$ fragments of 145-2C11 markedly reduced T cell activation, cytokine production, and morbidity /mortality while maintaining tolerance to skin grafts and blocking the induction of autoimmune diabetes (Hirsch *et al.*, 1990; Herold *et al.*, 1992). The mechanism of tolerance induction following $F(ab')_2$ treatment of mice was believed to be anergy since the provision of exogenous IL-2 restored the ability to reject skin grafts (Hirsch *et al.*, 1991).

Additional evidence which suggests that nonmitogenic hamster anti-mouse 145-2C11 $F(ab')_2$ antibodies induce anergy came from studies showing suppression of the development of collagen-induced arthritis (Hughes *et al.*, 1994). The mechanism for inducing T cell suppression is anergy induction in Th1-like (IL-2 and IFN-γ producing) cells but not Th2-like (IL-4) producing cells, as measured by *in vitro* restimulation responses to collagen. The failure to induce tolerance in the Th2 cells was supported by the finding that

IL-4 dependent auto-antibody production was not inhibited and the observations of others that IFN-γ exacerbates the arthritis, (Jacob *et al.*, 1987; Mauritz *et al.*, 1988), while IL-4 is protective (Marcelletti *et al.*, 1991). These observations are similar to those noted above for Th1 clones stimulated *in vitro* with anti-CD3 antibody in the absence of costimulation and support a two-signal model of T cell activation in which a FcγR-bearing cell is required for T cell activation and subsequent deletion but not anergy induction. My data suggest that there is either insufficient endogenous costimulation present at high doses of anti-CD3 antibody treatment to inhibit AICD or that AICD occurs independently of B7-dependent costimulation.

In vitro studies revealed an important role for the T cell costimulatory pathway in the sustained production of T cell cytokines and the prevention of anergy and apoptosis. The importance of costimulation for naive cells in these studies was found to be at least partly due to the production of IL-2 (DeSilva *et al.*, 1991; Harding *et al.*, 1992). Costimulation-dependent IL-2 production was also important for the production of IL-4 by naive cognate antigen-specific cells but *in vitro* IL-1 can substitute for CD28 signaling in Th2 clones for IL-4 production (Seder *et al.*, 1994; Lichtman *et al.*, 1988). The costimulation-dependent production of IL-4 was recently examined in response to infection with a gastrointestinal nematode *Heligmosomoides polygyrus*. The primary immune response to *H. polygyrus* included the B7-dependent production of IL-4 since T cell IL-4 production was prevented through the administration of CTLA4-Ig (Lu *et al.*, 1994). The *in vivo* production of IL-4 following low dose anti-CD3 antibody treatment is dependent on the endogenous costimulatory pathway as the chimeric fusion protein CTLA4-Ig was able to block IL-4

mRNA expression (Yoshimoto and Paul.1994). My data confirmed and extended this finding by demonstrating the production of the T cell cytokines IL-2 and IL-4 were dependent on endogenous costimulation following a low dose, but not high dose, treatment with anti-CD3 antibody. The expression of IL-2 and IL-4 could be significantly inhibited with the chimeric fusion protein CTLA4-Ig after low dose anti-CD3 antibody treatment (Figure 21). These data suggest that while IL-4 production does appear to require endogenous costimulation following weak TCR stimulation, a strong TCR signal will initiate IL-4 production independently of the costimulatory pathway.

The induction of T cell depletion through an activation-dependent mechanism was suggested to occur following TCR signaling in the absence of costimulation. Immunization of TCR transgenic mice with an immunogenic peptide can block induction of superantigen-induced apoptosis of the same cells, presumably through the induction of ligands for CD28/CTLA4 on APC (McCormack *et al.*, 1994). In addition, an *in vitro* study demonstrated that the induction of T cell apoptosis by religation of the TCR of previously activated T cells only occurred in the absence of costimulatory ligands but did not occur in the presence of costimulation (Radvanyi *et al.*, 1993). T cells from CD28 knockout mice could be stimulated *in vitro* with high dose anti-CD3 antibody but required ten-fold more antibody to initiate activation then wild type control cells, suggesting that at high levels of TCR signaling, costimulation becomes unnecessary (Green *et al.*, 1994). Therefore, the role of endogenous costimulation in the induction of AICD *in vivo* following high dose anti-CD3 antibody treatment was investigated to determine if costimulation plays a positive or negative role in the induction of AICD. Various doses of CTLA4-Ig were unable to prevent anti-CD3

antibody-induced T cell deletion or apoptosis (Figures 24). My results also indicate that blocking endogenous costimulation with CTLA4-Ig fails to prevent high dose anti-CD3 antibody-induced apoptosis (Figure 25 and Table 7). This suggests that if sufficient TCR ligation occurs, apoptosis will be the consequence regardless of the presence or absence of B7-dependent signaling. Alternatively, B7 expression may need further upregulation to provide sufficient signaling to rescue cells from high density TCR ligation.

Since LPS stimulation is known to up-regulate costimulatory molecules (Chen and Nabavi.1994) it was used to increase endogenous costimulatory molecules either before, concurrently, or after anti-CD3 antibody treatment. The addition of LPS failed to protect T cells from anti-CD3 antibody-induced apoptosis when given either before or after treatment, and, in fact, increased the anti-CD3 antibody-induced mortality when provided after anti-CD3 antibody treatment (Figures 26, 27). A noted exception are the results from two of three experiments demonstrating that pretreatment of mice with LPS in 6-12.5 μg range provided marginal protection from anti-CD3 antibody-induced T cell depletion. Although these data are intriguing, interpretation of the results is difficult. These results could be due to LPS-induced tolerance or reflect an alteration in the kinetics of T cell depletion in the presence of a specific dose of LPS. Additional studies must be performed to identify the basis of this result. The weight of these findings suggest that LPS up-regulation of costimulatory molecules is unable to prevent anti-CD3 antibody-induced apoptosis. Alternatively, it could be possible that the additional systemic inflammation that LPS induces masks any potential benefits that costimulation may provide for T cell survival. It was recently shown that LPS rescued T cells from superantigen-induced deletion *in vivo*

apparently through a B7-independent, TNF-α-dependent mechanism (Vella *et al.*, 1995). One possible reason for LPS rescue of T cell depletion in SEB-treated mice, but not anti-CD3 antibody treated mice, may be the difference in the density of TCR ligation or additional signals induced by SEB through MHC class II molecules . Taken together, the results from my thesis suggest that following high dose stimulation of the TCR, costimulatory signals are unable to positively or negatively influence AICD, implying an important role for the strength or quantitative level of TCR signaling in determining the fate of an activated T cell.

Quantitative differences in TCR signaling lead to different outcomes of T cell activation. Important considerations in the initiation of apoptosis include the strength of the stimulating signal, the presence of costimulation, the proper timing, and the combination of cell surface and cytokine-mediated accessory signals (Kabelitz *et al.*, 1993; Russell *et al.*, 1991; Perandones *et al.*, 1993; Boehme and Lenardo.1993; Radvanyi *et al.*, 1993; Critchfield *et al.*, 1994; Janeway and Bottomly.1994). Selection of T cells in the thymus and deletion of T cells by bacterial or endogenous superantigens provide support for the hypothesis that the fate of a mature T cell can be altered by the strength of the signal initiated through the TCR. In the case of thymocytes undergoing selection, a strong interaction with MHC molecules can lead to negative selection, while a weaker interaction to positive selection of the responding T cell (Sebzda *et al.*, 1994). These findings were extended in studies with LCMV peptide-specific transgenic mice showing that the strength of signal-one delivered to thymic T cells was dependent on the number of T cell receptors engaged: low

numbers led to positive selection while high numbers led to negative selection (Ashton-Rickardt *et al.*, 1994). Mature peripheral T cells become activated and develop immunological memory when stimulated with a low dose of a strong antigen but will become tolerized when exposed to a high dose of the same antigen. This phenomenon was studied in an autoimmune model of experimental autoimmune encephalomyelitis (EAE) in which low doses of myelin basic protein (MBP) initiate EAE but high doses of MBP abrogate EAE and induce deletion of autoreactive T cells (Critchfield *et al.*, 1994). The basis for this differential response may be due to the quantitative signal these cells receive.

Models with superantigens also provide evidence that the quantitative signal a T cell receives is important because the deletion of T cells following exposure to superantigen is dose dependent (Jones *et al.*, 1990), and occurs independently of B7-dependent costimulation (Damle *et al.*, 1993). The differential response of anergy or apoptosis following *in vivo* treatment with the superantigen SEB is based primarily on the degree of T cell activation: T cells which proliferate in response to SEB undergo apoptosis while those responsive cells which do not proliferate become anergic (Renno *et al.*, 1995). These findings suggest that a quantitative difference in TCR signaling may determine the fate of peripheral T cells. My findings suggest that peripheral T cells undergo a dose dependent AICD when treated with 5-30 μg of anti-CD3 antibody. Based on the assumption that high dose anti-CD3 antibody treatment is equivalent to a high number of TCR signaling events, the higher doses of anti-CD3 antibody may induce AICD because they initiated a T cell response similar to high zone suppression.

A form of T cell tolerance, termed high zone suppression, can occur if mice are administered high doses of an antigen. High zone suppression induced by large doses of TNP-OVA is directed at Th1-like cells which became anergic (Burstein and Abbas.1993). The Th2-like cells were refractory to this form of tolerance induction, and the IL-4 that they produced was hypothesized to suppress IL-2 production by the Th1 cells. These findings suggest the importance of the antigen dose in regulating T cell responsiveness *in vivo*. Although the induction of anergy by anti-CD3 antibody was not assessed in my experiments. it is possible that an anti-CD3 antibody-mediated high zone tolerance model includes both anergy and AICD as mechanisms to induce unresponsiveness of peripheral T cells.

Data from *in vitro* studies on anti-CD3 antibody treatment of nontransformed T cell clones demonstrated that quantitative differences in TCR signaling alternatively induce proliferation or apoptosis. The outcome was based on differences in the amount of TCR ligation and the dose of exogenous IL-2, suggesting that apoptosis can be induced by the relative strength of the activation signals (Ucker *et al.*, 1992). The finding that $CD4^-$ $NK1.1^+$ cells were considerably more resistant to anti-CD3 antibody-induced apoptosis than $CD4^+$ $NK.1.1^+$ cells (Table 6), suggests that this population may be uniquely suited to respond to antigen at early stages of the immune response before costimulatory molecules are elevated to optimal levels for T cell signaling. Furthermore, this property would be important if this early responding cell population does play an important role in influencing the development of the Th2 response, as recently suggested (Bluestone.1995). The basis of the resistance of the $CD4^-$ $NK1.1^+$ subset to anti-CD3 antibody-induced apoptosis may be the low expression of TCR-CD3 complexes on the cell surface . The apoptotic process may be initiated on the

quantitative strength of the signal delivered through the TCR, and low CD3 expression may consequently protect $CD4^-$ $NK1.1^-$ cells from AICD. This hypothesis is supported by my observation that doses of anti-CD3 antibody below 5 μg did not initiate detectable T cell depletion or apoptosis of peripheral $CD4^-$ T cells. Alternatively, other unidentified properties of this $CD4^-$ $NK1.1^-$ T cell population may contribute to its increased resistance to apoptosis.

The apoptotic phenotype and expression of apoptosis-associated genes. Mice with defects in apoptosis *(lpr* and *gld)* accumulate an unusual set of lymphocytes that are $Thy1^-$ $B220^-$ $CD4^-$ $CD8^-$ (Cohen *et al.*, 1992). The basis for the accumulation of these cells is unknown but may be due to defects in the apoptotic pathway. I hypothesized that this cell population may represent a general preapoptotic phenotype that accumulates in *fas*-defective mice. Therefore, the surface phenotype of cells induced by anti-CD3 antibody to undergo apoptosis was examined. Initial studies demonstrated that anti-CD3 antibody treated cells had greatly reduced TCR staining probably because of TCR modulation following anti-CD3 antibody binding or direct interference of anti-TCR antibody binding by anti-CD3 antibody. The apoptotic cells in this experiment also had an intermediate Thy1 labeling pattern suggesting those apoptotic T cells had decreased Thy1 expression or that apoptotic T cells were nonspecifically binding the anti-Thy1 antibody (Figure 8). A control antibody of the same isotype as the anti-Thy1 antibody did not bind the apoptotic cells suggesting that nonspecific binding was not the reason for the Thy1 intermediate phenotype. The down-regulation of cell surface molecules on apoptotic cells may be due to a general degradation

of macromolecules or may be due to the described changes in the plasma membrane known to occur in cells undergoing apoptosis (Cohen *et al.*, 1992). It was shown that thymocytes undergoing apoptosis in culture down-regulate surface CD4 and CD8 expression and up-regulate TCRβ/CD3, CD69 and IL-2R (CD25) expression (Kishimoto *et al.*, 1995). My results also showed that apoptotic cells with reduced FSC were negative for the T cell surface markers CD4 and CD8 and positive for the B cell marker B220 and the T cell surface marker Thy1 (Figure 9-13 and Tables 4, 5). These data suggest that, as in the thymus, T cells in the periphery down-regulate surface expression of CD4 or CD8 and initially increase expression of IL-2R when undergoing apoptosis. In addition, the expression of Thy1 is down-regulated with the novel expression of the B cell surface marker B220. The phenotype of the majority of T cells undergoing anti-CD3 antibody-induced apoptosis ($Thy1^-$ $B220^+$ $CD4^-$ $CD8^-$) is strikingly similar to T cells which accumulate in autoimmune *lpr* mice, and thus may represent a general apoptotic T cell surface phenotype. The expression of B220 in T cells undergoing apoptosis *in vivo* may initiate down-regulation of an immune response by marking T cells as "aberrant" and thus signal their removal. Alternatively, as the need for activated T cells decreases at the end of an immune response and T cells fail to receive continued stimulation, they may be triggered to undergo apoptosis. As part of this process, T cells may lose regulatory control of their cell surface marker expression (*i.e.*, down-regulate CD4 and up-regulate B220). The similarity of the cell surface phenotype of peripheral T cells which accumulate in *lpr* and *gld* mice and apoptotic cells which are observed in mice following treatment with anti-CD3 antibody suggests the involvement of the *fas/fasL* pathway in peripheral T cell apoptosis.

The T cell surface phenotype observed following treatment with anti-CD3 antibody. ($Thy1^+$ $B220^+$ $CD4^-$ $CD8^-$), was detected in *lpr* and *gld* mice which exhibit lymphadenopathy associated with the accumulation of large numbers of these lymphocytes (Takahashi *et al.*, 1994; Nagata and Golstein.1995). These mice have mutations in the *fas* and *fasL* genes respectively. *Fas* and *fasL* play a role in AICD and it was hypothesized that the large numbers of aberrant ($Thy1^+$ $B220^+$ $CD4^-$ $CD8^-$) lymphocytes that accumulate in these mice are a result of a failure of lymphocytes to undergo a *fas* dependent AICD (Russell *et al.*, 1993). The observation that this rare phenotype is increased in anti-CD3 antibody treated mice suggests the possibility that it is a characteristic of cells committed to undergo AICD. Whether these cell surface molecules have particular functions during the apoptotic process or are ancillary should be determined in future studies.

T lymphocytes from *lpr* mice are resistant to high dose anti-CD3 antibody-induced anergy and apoptosis (Bossu *et al.*, 1993; Watanabe-Fukunaga *et al.*, 1992; Gillette-Ferguson and Sidman.1994). Conversely, it was shown that these cells undergo a rapid apoptotic cell death when incubated *in vitro* for four h without additional stimulation which suggests a *fas*-independent apoptotic pathway that may be repressed *in vivo* (Van Houten and Budd.1992). The nature of this repression is unclear but could reflect an uncoupling of the TCR from the apoptotic pathway due to the absence of *fas*. The T cell depletion resulting from anti-CD3 antibody-induced apoptosis may reflect a mechanism of peripheral T cell tolerance that occurs in response to self-antigens. Autoreactive T cells may escape thymic negative selection and upon entering the periphery may encounter self-antigens presented by APCs. If costimulatory molecules are available then T cell activation leading to cytokine

production would be expected to occur. Conversely, if TCR signaling occurs in the absence of costimulation, T cell AICD could result. Since self-antigens are not associated with agents, such as LPS, that induce expression of APC costimulatory molecules, they would be expected to induce AICD when recognized be autoreactive T cells, resulting in the deletion of these T cells that could otherwise promote autoimmune disease. Although my studies do not directly address T cell autoreactivity, they are a model of T cell signaling via the TCR. Clearly my findings suggest that T cell activation can lead to dose-dependent cytokine production, which can be B7-dependent. They also demonstrate that high density TCR occupancy can lead to AICD regardless of B7-dependent signaling. These results suggest that there may be an additional mechanism to delete autoreactive T cells: a strong TCR mediated signal induced by self-peptides which are abundantly expressed on cells, which does not require B7-dependent costimulation. This mechanism may be dependent on apoptosis-inducing pathways such as *fas* mediated signaling.

A defect in *fas* expression may block AICD promoting T cell autoreactivity and autoimmune disease. The single gene *fas* defect in *lpr* mice is associated with autoimmunity and lymphadenopathy (Takahashi *et al.*, 1994; Nagata and Golstein. 1995). Recent evidence suggests that some patients with serum lupus erythematosus (SLE) may also have defects in the *fas* gene (Cheng *et al.*, 1994). Because of the importance of *fas* in mediating apoptosis, *fas* and *fasL* expression in T cells of mice treated with anti-CD3 antibody was examined. Anti-CD3 antibody treatment was shown to up-regulate expression of *fas* and *fasL* mRNA early in $Thy1^+$ cells, and later an increase in cell surface expression of *fas* was detected in both $CD4^+$ and $CD8^+$ cells (Figures 14-16). The increased expression of *fas* and *fasL* in T

cells prior to the initiation of apoptosis suggests that they may play an important role in the reponse to a strong TCR signal which may result in the removal of inappropriately stimulated peripheral T cells.

To determine the role of *fas*-mediated signaling in anti-CD3 antibody-induced apoptosis experiments using *lpr* mice were initiated. Although the experiments were not completed, preliminary data indicated anti-CD3 antibody treatment of *lpr* mice initiated apoptosis and depletion of $CD4^-$ and $CD8^+$ cells, and an increase in $B220^-$ $Thy1^-$ apoptotic cells. T cells in these mice were able to undergo apoptosis, although perhaps not at the same rate as wild type controls. A recently published article has confirmed my initial findings by demonstrating comparable *in vivo* apoptosis of MRL-*lpr/lpr* and control MRL-+/+ lymph node T cells following high dose (50 μg) anti-CD3 antibody but dissimilar apoptosis following low dose (10μg) anti-CD3 antibody treatment (Tucek-Szabo *et al.*, 1996). Similar results are described in experiments with transgenic mice expressing a rearranged TCR for the H-Y antigen and carrying the *lpr* mutation in which autoreactive cells in male mice were observed to undergo clonal deletion (Zhou *et al.*, 1991). Also, *lpr* mice were demonstrated to be competent in response to the superantigen SEB, in that peripheral $V\beta8^-$ cells were found to be anergic upon *in vitro* restimulation although the extent of *in vivo* deletion was somewhat reduced compared to wild type controls (Scott *et al.*, 1993). These observations and my results suggest that there may be alternative pathways to delete hyper-activated or autoreactive cells that are not *fas*-mediated. Further, my data do not exclude the involvement of *fas*-mediated apoptosis following anti-CD3 antibody signaling.

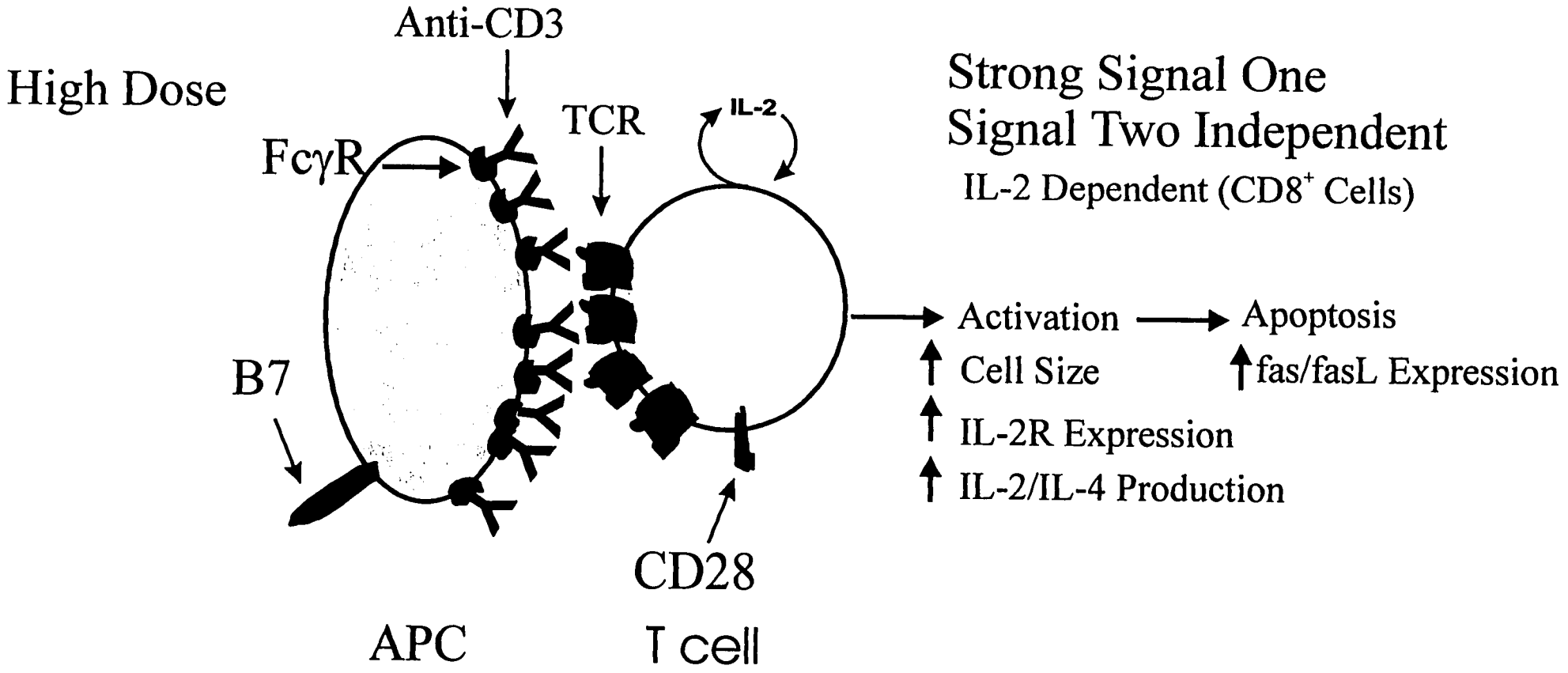
A possible trigger for AICD could be decreased *bcl-2* expression, an inhibitor of apoptosis. It was previously demonstrated that memory T cells isolated from patients with Herpes virus infections or HIV infections rapidly undergo apoptosis *in vitro*, and this apoptosis is correlated with low expression of the *bcl-2* gene (Akbar *et al.*, 1993). *bcl-2* knockout mice have massive apoptotic involution of both the thymus and spleen which becomes progressive with age and lymphocyte activation suggesting a role for *bcl-2* in preventing activation-induced apoptosis (Veis *et al.*, 1992). Lymphocytes from transgenic mice which over-express *bcl-2* have an increased resistance to anti-CD3 antibody-induced deletion *in vitro*. These mice do not accumulate large numbers of peripheral lymphocytes *in vivo* suggesting that *bcl-2* promotes an activation-independent survival mechanism (Akbar *et al.*, 1993). My findings show no increase in *bcl-2* mRNA following anti-CD3 antibody treatment and may indicate a decrease in expression, suggesting that reduced *bcl-2* expression may contribute to anti-CD3 antibody-induced apoptosis (Figure 14).

Collectively, these results suggest that anti-CD3 antibody-induced apoptosis may occur by a *fas*-independent mechanism which is augmented by *fas* signaling. Alternatively, since the *lpr* mutation is leaky, very low levels of *fas* expression may be sufficient to trigger apoptosis induced by anti-CD3 antibody in *lpr* mice (Watanabe-Fukunaga *et al.*, 1992). There is also the possibility that in *lpr* mice, compensatory mechanisms not utilized when *fas* is normally expressed, may mediate apoptosis. To resolve this dilemma, blockage of surface *fas* signaling in *lpr* and wild type mice following anti-CD3 antibody treatment, with a chimeric FAS-Fc construct, should be preformed in future studies (Cheng *et al.*, 1994).

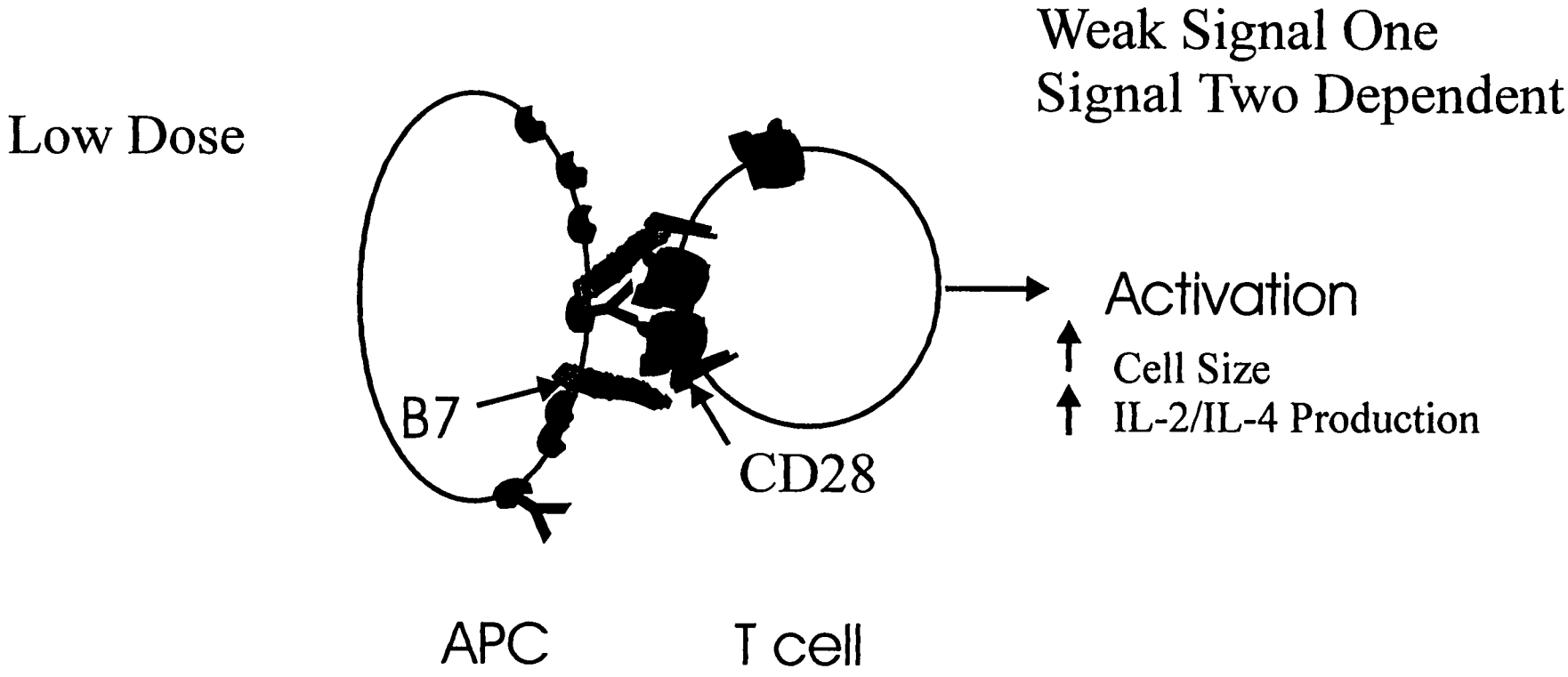
Model. A proposed model of T cell interactions involved in activation at high versus low density TCR cross-linking by anti-CD3 antibody is depicted in Figures 29-30. High density anti-CD3 antibody binding causes T cell deletion through apoptosis. A FcγR-bearing APC binds anti-CD3 antibody which then cross-links the TCR/CD3 complex leading to T cell activation. The high density of TCR ligation on the T cell is sufficient to support rapid differentiation to cytokine secretion in the absence of B7-dependent costimulatory signals. During the first 24 h, $CD4^+$ and $CD8^+$ cells become activated, as measured by increased cell size and IL-2R expression. These T cells, in particular $CD4^+$ $NK1.1^-$ T cells, rapidly produce cytokines including IL-2 and IL-4 both before and during measurable increases in markers of activation. One to four days following treatment with anti-CD3 antibody the T cell surface phenotype changes with down regulation of CD4, CD8 and Thy1 and upregulation of B220. These cells subsequently undergo apoptosis, associated with increased expression of both *fas* and *fasL* mRNA and surface expression of Fas protein. The $CD4^+$ $NK1.1^-$ cells appear to be much more resistant to AICD. The functional contribution of increased *fas* and *fasL* expression to anti-CD3 antibody induced apoptosis in this model is unclear. Cytokine requirements for AICD differ in $CD8^+$ and $CD4^+$ T cells in that only the former requires IL-2 signaling.

Treatment with low doses of anti-CD3 antibody (0.1 μg) also lead to cytokine production. But at this lower density of TCR cell surface cross-linking, B7-dependent costimulation is also required. At this lower dose a smaller increase in cell size is observed for both $CD4^+$ and $CD8^+$ T cells, no increase is observed in IL-2R expression and AICD as measured by depletion and apoptosis is not detectable.

Proposed Model for The In Vivo Effects of High Dose Anti-CD3 Antibody on Peripheral T Cells



Proposed Model for The In Vivo Effects of Low Dose Anti-CD3 Antibody on Peripheral T Cells



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